

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
6 June 2002 (06.06.2002)

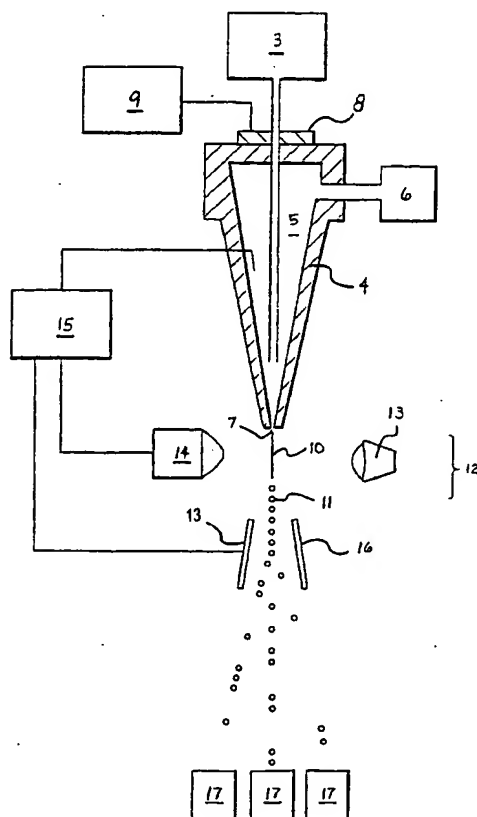
PCT

(10) International Publication Number  
**WO 02/43574 A2**

- (51) International Patent Classification<sup>7</sup>: **A61B** US 60/253,787 (CON)  
Filed on 29 November 2000 (29.11.2000)
- (21) International Application Number: PCT/US01/45023
- (22) International Filing Date: 29 November 2001 (29.11.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
60/253,785 29 November 2000 (29.11.2000) US  
60/253,787 29 November 2000 (29.11.2000) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:  
US 60/253,785 (CON)  
Filed on 29 November 2000 (29.11.2000)
- (71) Applicants (for all designated States except US): **XY, INC.** [US/US]; 1108 North Lemay Avenue, Fort Collins, CO 80524 (US). **COLORADO STATE UNIVERSITY, through its agent, COLORADO STATE UNIVERSITY RESEARCH FOUNDATION** [US/US]; Room 410, 601 South Howes Street, Fort Collins, CO 80522 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **SEIDEL, George, E.** [US/US]; XY, Inc., 1108 North Lemay Avenue, Fort Collins, CO 80524 (US). **SUH, Tae Kwang** [US/US]; XY, Inc., 1108 North Lemay Avenue, Fort Collins, CO 80524 (US). **LU, Kehuan** [US/US]; XY, Inc., 1108 North Lemay Avenue, Fort Collins, CO 80524 (US).

[Continued on next page]

(54) Title: SYSTEM TO SEPARATE FROZEN-THAWED SPERMATOZOA INTO X-CHROMOSOME BEARING AND Y-CHROMOSOME BEARING POPULATIONS



(57) Abstract: Devices, compositions, and methods for handling, separating, packaging, and utilization of spermatozoa (1) that can be derived from previously frozen sperm samples collected from a male mammel. Specifically, techniques to uniformly stain (2) spermatozoal DNA even when derived from previously frozen sperm and separation techniques to separate and isolate spermatozoa even when derived from previously frozen sperm samples into X-chromosome bearing and Y-chromosome bearing populations having high purity.

WO 02/43574 A2

- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## SYSTEM TO SEPARATE FROZEN-THAWED SPERMATOZOA INTO X-CHROMOSOME BEARING AND Y-CHROMOSOME BEARING POPULATIONS

This application claims the benefit of United States Provisional Patent Application  
5 No. 60/253,787, filed November 29, 2000 and United States Provisional Patent Application  
No. 60/253,785, filed November 29, 2000, each hereby incorporated by reference herein.

### I. TECHNICAL FIELD

The invention involves the substantially uniform binding of fluorochrome(s) to the  
10 DNA within mammalian spermatozoa (or sperm cells) allowing such labeled spermatozoa to  
be separated into high purity X-chromosome bearing and Y-chromosome bearing  
populations. Specifically, methods for the substantially uniform binding of fluorochrome(s)  
to the DNA of mammalian spermatozoa contained within previously frozen and then thawed  
semen. In addition, the invention further involves devices, methods, and compositions for  
15 the use of high purity separated X-chromosome bearing and Y-chromosome bearing  
populations of spermatozoa from previously frozen-thawed semen in processes involving, but  
not limited to, artificial insemination, surgical insemination, and in-vitro fertilization and  
embryo culturing techniques.

### 20 II. BACKGROUND

Sperm can be collected from a great variety of mammals and then separated into X-  
chromosome bearing and Y-chromosome bearing populations based upon the difference in  
DNA content. In some conventional methods of spermatozoa separation, the DNA content of  
the spermatozoa to be separated can be stained with a fluorochrome(s) that upon excitation  
25 emit(s) a measurable amount of fluorescence. Because X-chromosome bearing spermatozoa  
contain a greater amount of DNA than Y-chromosome bearing spermatozoa, each X-  
chromosome bearing spermatozoa has the capacity to bind a relatively greater amount of  
fluorochrome than the corresponding Y-chromosome bearing spermatozoa. Comparison of  
the relative magnitude of emitted fluorescence upon excitation of the fluorochrome(s) allows  
30 the isolation of X-chromosome bearing spermatozoa from Y-chromosome bearing  
spermatozoa as described by United States Patent No. 5,135, 759, hereby incorporated by  
reference.

Even though X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa have been differentiated by and separated based upon the difference in emitted fluorescence for many years, and even though there is large commercial market for isolated populations of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa, there remain significant problems yet to be resolved.

A significant problem with conventional methods of separating X-chromosome bearing spermatozoa from Y-chromosome bearing spermatozoa can be that each resulting population contains a significant number of incorrectly separated spermatozoa that belong in the other population. This problem in differentiating between spermatozoa can, in part, be attributed to the lack of uniformity in the amount of fluorochrome bound to the spermatozoal DNA. As such, a range in the amount of fluorochrome bound by X-chromosome bearing spermatozoa is generated and a range in the amount of fluorochrome bound by Y-chromosome bearing spermatozoa is generated. When these ranges in the amount of fluorochrome overlap or yield some values that are similar, it can be difficult or impossible to classify those individual spermatozoa to one population or the other with any degree of certainty and cross contamination of the populations can occur.

This particular problem can be exacerbated with regard to spermatozoa obtained from frozen and subsequently thawed mammalian semen. The mean purity for separated Y-chromosome bearing spermatozoa population derived from previously frozen-thawed semen can be 85% or less, and the mean purity for separated X-chromosome bearing spermatozoa population derived from previously frozen-thawed semen can be 82% or less.

Another significant problem associated with staining of spermatozoal DNA can be the detrimental effects on fertilization rates and subsequent embryonic development of fertilized oocyte(s) (oocyte, ootid, or ovum, or a plurality of same, as may be appropriate within a specific application). One aspect of this problem may be that the amount of stain bound to the DNA may effect the viability of the spermatozoa resulting in lower fertilization rates. Another aspect of this problem can be that the amount of time that elapses during the staining of the DNA may effect the viability of the sperm resulting in lower fertilization rates.

Another aspect of this problem may be that the amount of time that elapses during staining of the DNA may lower subsequent cleavage rates of oocytes fertilized with such stained spermatozoa. A 20% decline in cleavage rates have been observed for oocytes when staining time requires 190 minutes as compared to when staining time requires 60 minutes. Another  
5 aspect of this problem may be that the percent of oocytes fertilized with stained spermatozoa that proceed to blastulation may be lower as described in the journal article entitled "In vitro Fertilization with Flow-Cytometrically-Sorted Bovine Sperm", Theriogenology 52: 1393-1405 (1999), hereby incorporated by reference herein.

10 Another significant problem may be that cryopreserved sperm may demonstrate increased capacitation, and the length of time such spermatozoa are viable may be shortened. As such, if previously frozen spermatozoa are to be separated into X-chromosome bearing and Y-chromosome bearing populations that are to be subsequently used in applications such as in-vitro fertilization, in-vivo artificial insemination, or the like, then routine staining  
15 procedures may have to be abbreviated to maintain suitable number of viable sperm cells.

As relating to the problems of staining spermatozoa uniformly, even when spermatozoa are obtained from previously frozen-thawed semen; maintaining sperm viability; separating stained spermatozoa into X-chromosome bearing and Y-chromosome bearing  
20 populations, even when the spermatozoa being separated are obtained from previously frozen semen; generating populations of X-chromosome bearing and Y-chromosome bearing spermatozoa having high purity; and successfully using separated spermatozoa for artificial insemination, surgical insemination, and in-vitro fertilization techniques it can be understood there are significant problems with conventional technology which are addressed by the  
25 instant invention.

### III. DISCLOSURE OF THE INVENTION

A broad object of embodiments of the invention can be to provide DNA staining  
30 technology that allows substantially uniform amounts of fluorochrome to be bound to the DNA of all individual spermatozoa bearing an X-chromosome and substantially uniform

amounts of fluorochrome to be bound to all individual spermatozoa bearing a Y-chromosome within an amount of semen.

One aspect of this broad object of the invention can be to narrow the range in  
5 magnitude of emitted fluorescence for each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa upon passing through a fluorochrome excitation source.

Another aspect of this broad object of the invention can be to increase the difference  
10 between the mean values of magnitude of emitted fluorescence for each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa upon passing through a fluorochrome excitation source.

Another aspect of this broad object of the invention can be to decrease the number  
15 of spermatozoa incorrectly assigned to each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa.

Another aspect of this broad object of the invention can be to generate separate X-chromosome bearing and Y-chromosome bearing populations having greater than 85%  
20 purity or greater than 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or even 99% purity.

Another broad object of embodiment of the invention can be to allow assessment of a wide range of genetics. Rather than being limited to the genetics of individuals from species  
25 of mammals having proximity to a spermatozoa separating or sorting facility, genetics representing a wide variety of individuals from numerous species can be transported as frozen semen to distant spermatozoa separation facilities for subsequent separation into X-chromosome bearing and into Y-chromosome bearing populations. These species of mammals may include, but are not limited to primates, such as chimpanzees, gorillas,  
30 humans, or the like; marine mammals, such as whales, porpoises, or the like; bovids; ovids; swine; canids; felids; or equids, as but a few examples. It may also include genetics that are

considered rare because the species of mammal may be endangered or few in number; or considered rare because the individual has desirable morphological, physiological, or intellectual attributes.

5           Another broad object of embodiments of the invention can provide separation technology for differentiating between X-chromosome bearing and Y-chromosome bearing spermatozoa obtained from frozen-thawed semen.

10           Another object of embodiments of the invention can be to provide DNA staining technology to more uniformly stain the DNA of spermatozoa contained in frozen-thawed semen to improve the apparent resolution between X-chromosome bearing and Y-chromosome bearing spermatozoa.

15           Another object of embodiments of the invention can be to provide high purity artificial insemination samples prepared from separated spermatozoa from frozen-thawed semen.

20           Another object of embodiments of the invention can be to provide high purity low dose artificial insemination samples prepared from separated spermatozoa from frozen-thawed semen.

25           Another object of embodiments of the invention can be to provide high purity insemination samples for surgical insemination procedures prepared from separated spermatozoa from frozen-thawed semen.

          Another object of an embodiment of the invention can be to provide high purity insemination samples for in-vitro fertilization procedures prepared from separated spermatozoa from frozen-thawed semen.

30           Another object of an embodiment of the invention can be to provide high purity insemination samples for in-vitro fertilization procedures prepared from separated

spermatozoa from frozen-thawed human semen.

Another object of an embodiment of the invention can be to provide technology for staining and separation of spermatozoa from frozen-thawed sperm into X-chromosome bearing populations and Y-chromosome bearing populations for in-vitro fertilization of oocyte(s) that is not detrimental to cleavage rates or embryonic development.

Naturally further objects of the invention are disclosed throughout other areas of specification.

10

#### IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a particular embodiment of the invention for staining the DNA of spermatozoa contained in frozen-thawed semen.

15

Figure 2 shows a particular embodiment of the invention for separating spermatozoa from frozen-thawed semen into X-chromosome bearing and Y-chromosome bearing spermatozoa.

20

Figure 3 shows a further view of a particular embodiment of the invention for separating spermatozoa from frozen-thawed semen into X-chromosome bearing and Y-chromosome bearing spermatozoa.

#### V. MODE(S) FOR CARRYING OUT THE INVENTION

To routinely separate spermatozoa (live, fixed, viable, non-viable, or nuclei) into high purity X-chromosome bearing samples and into Y-chromosome bearing samples, the method used to sort the X-chromosome bearing and Y-chromosome bearing spermatozoa must provide sufficient resolution of the X-chromosome bearing spermatozoa from the Y-chromosome bearing spermatozoa so that separation or sorting step(s) can be achieved without substantial cross contamination.

30

Resolution or differentiation of spermatozoa can be based upon ascertaining the



difference in the fluorescent emission from the amount of fluorochrome bound to the DNA within the X-chromosome bearing spermatozoa upon excitation and the fluorescent emission from the amount of fluorochrome bound to the DNA within the Y-chromosome bearing spermatozoa upon excitation. Separation of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa based upon this measurable difference may then be achieved by a number of methods such as flow cytometry, liquid chromatography, gel electrophoresis, and other technologies that similarly compare the relative magnitude of fluorescence to differentiate between X-chromosome bearing spermatozoa and the Y-chromosome bearing spermatozoa.

10

Spermatozoa separation systems can have problems differentiating between the fluorescent emission generated by the fluorochrome bound to the DNA of X-spermatozoa, and the fluorescent emission generated by the fluorochrome bound to the DNA of Y-spermatozoa upon excitation when the amount of the fluorochrome bound to the DNA of individual spermatozoa is not consistent within the Y-chromosome bearing or X-chromosome bearing populations. These difficulties in differentiating between the amount of fluorescent emissions generated by the bound fluorochrome(s) become exacerbated when spermatozoa are obtained from frozen-thawed sperm which are stained by conventional techniques.

20

The failure to stain the spermatozoal DNA consistently can generate a broader range of fluorescing species for both X-chromosome bearing and Y-chromosome bearing populations of spermatozoa. This broader range of fluorescing species for the two populations results in an increased range of apparent DNA molecular weights and a decreased ability to resolve X-chromosome bearing from Y-chromosome bearing spermatozoa. The decrease in resolution makes separation of the X-chromosome bearing spermatozoa from the Y-chromosome bearing spermatozoa more difficult and results in cross contamination between populations and a lower purity of separated spermatozoa samples are obtained.

30

Particular embodiments of the invention provide technology to stain the DNA of live

viable spermatozoa or the spermatozoal DNA of frozen-thawed semen specimens to allow increased resolution of X-chromosome bearing from the Y-chromosome bearing spermatozoa resulting in high purity X-chromosome bearing and high purity Y-chromosome bearing populations of sperm cells. As such, it is understood that the term high purity can mean  
5 greater resolution of the X-chromosome bearing from the Y-chromosome bearing spermatozoa compared to conventional staining technology for a given application. High purity can also mean less cross contamination between separated spermatozoa populations compared to conventional separation technologies.

10 For example, in particular flow cytometry embodiments of the invention, high purity for stained frozen-thawed live spermatozoa can mean sorted populations of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa having a purity greater than about 85%. However, if live viable sperm or sperm nuclei are being sorted high purity may mean X-chromosome bearing and Y-chromosome bearing populations having a purity greater  
15 than about 90%. As can be understood, the definition of high purity is contextual involving a comparison of the results obtained from each embodiment of the invention compared to the results obtained when utilizing convention technologies for a particular application. In the context of spermatozoa having DNA that stains poorly, such as previously frozen-thawed spermatozoal DNA, high purity can mean populations of isolated spermatozoa bearing  
20 greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of either an X-chromosome or a Y-chromosome.

Embodiments of the invention can include spermatozoa collected from numerous species of male mammals, and the invention should be understood not to be limited to the  
25 species of male mammals described by the specific examples within this application. Rather the specific examples within this application are intended to be illustrative of the varied and numerous species of male mammals from which semen can be collected and utilized in certain embodiments of the invention. Embodiments of the invention, for example, may include the spermatozoa of animals having commercial value for meat or dairy production  
30 such as swine, ovids, bovids, equids, buffalo, or the like (naturally the mammals used for meat or dairy production may vary from culture to culture). It may also include the

spermatozoa of various domesticated mammalian species encompassed by canids and felids. It may also include spermatozoa from individuals of various mammalian species that have uncommon attribute(s), such as morphological characteristics including weight, size, or conformation, or other desired characteristics such as speed, agility, intellect, or the like. It may also include spermatozoa of primates, including but not limited to chimpanzees, gorillas, or humans and the spermatozoa from marine mammals such as whales and dolphins. It may also include frozen-thawed spermatozoa from all the various mammals above-described and further, including but not limited to, the spermatozoa of deceased donors, from rare or exotic mammals, zoological specimens, or endangered species.

10

Now referring primarily to Figure 1, particular embodiments of the invention can comprise semen containing spermatozoa (1) collected from a male mammal, including but not limited to, those above-described. The spermatozoa can be incubated in a concentration of Hoechst 33342 stain (2) of greater than about 40  $\mu\text{M}$  at a temperature between about 30 ° Centigrade and about 40° Centigrade for a duration of time between 50 minutes to 200 minutes to stain spermatozoal DNA with sufficient uniformity to allow X-chromosome bearing spermatozoa to be differentiated from Y-chromosome bearing spermatozoa based upon the magnitude of fluorescence at a rate greater than about 85%.

20 The concentration of Hoechst 33342 stain between 40  $\mu\text{M}$  and 2500  $\mu\text{M}$  , the temperature between 30 ° Centigrade and about 40° Centigrade, and the duration of time between 50 minutes and 200 minutes can be selected to adjust the purity of the separated X-chromosome bearing and Y-chromosome bearing populations, or can be selected to promote cleavage rates and embryonic development, as further discussed below.

25

For example, when staining spermatozoal DNA from certain bovine species, the concentration of Hoechst 33342 can be increased to between about 200  $\mu\text{M}$  and about 2500 $\mu\text{M}$ , incubated for a period of time between about 60 minutes to about 190 minutes at a temperature of about 37 ° Centigrade. Specifically with respect to certain frozen-thawed bovine spermatozoa, the Hoechst 33342 stain (2) can be adjusted to establish a concentration of 2240 $\mu\text{M}$  and then incubated for about 60 minutes at about 39 ° Centigrade.

30

With respect to the cleavage rates of oocytes inseminated with mammalian sperm cells treated according to the invention, the increase in stain concentration up to at least 2240 $\mu$ M does not appear to have a depressive effect on either cleavage or embryonic development. Higher stain concentrations may actually be beneficial with respect to certain  
5   embodiments of the invention because the length of incubation time may be decreased improving percent cleavage or blastocyst formation. From application to application the concentration of Hoechst 33342, the length of incubation time, or both can be adjusted to obtain the maximal cleavage rate and blastocyst formation, if desired.

10       Now referring primarily to Figures 2 and 3, flow cytometric embodiments of the invention can include a cell source (3) which acts to establish or supply stained spermatozoa (fresh, frozen-thawed, sperm nuclei, or the like) to be analyzed by flow cytometry. The cells are deposited within a nozzle (4) in a manner such that the stained sperm cells are surrounded by a sheath fluid (5). The sheath fluid (5) is usually supplied by a sheath fluid source (6) so  
15   that as the cell source (3) supplies sperm cells, the sheath fluid (5) is concurrently fed through the nozzle (4). In this manner the sheath fluid (5) forms a sheath fluid environment for the sperm cells. Since the various fluids are provided to the flow cytometer at some pressure, they flow out of the nozzle (4) and exit at the nozzle orifice (7). By providing a type of oscillator (8) which may be very precisely controlled through an oscillator control (9),  
20   pressure waves may be established within the nozzle (4) and transmitted to the fluids exiting the nozzle (4) at the nozzle orifice (7). Since the oscillator (9) acts upon the sheath fluid (5), the stream (10) exiting the nozzle orifice (7) eventually and regularly forms drops (11). Because the sperm cells are at least partially surrounded by a sheath fluid environment, the drops (11) can contain within them individually isolated sperm cells.

25

Since the drops (11) generally contain individual isolated sperm cells, the flow cytometer can distinguish and separate droplets based upon the magnitude of fluorescence emitted from the fluorochrome bound to the spermatozoal DNA. This is accomplished through a cell sensing system (12). The cell sensing system involves at least some type of  
30   sensor (13) which responds to the magnitude of fluorescence emitted by each sperm cell contained within each drop (11). The sperm cell sensing system (13) may cause an action

depending upon the relative presence or relative absence of fluorescence emitted by the bound fluorochrome upon excitation by some stimulant such as the laser exciter (14). While each spermatozoon can be stained by the fluorochrome, such as Hoechst 33342, as described above, the differing amount of DNA comprising the X-chromosome and the Y-chromosome  
5 causes different amounts of stain to be bound. Thus, by sensing the degree of fluorescence emitted by the fluorochrome upon excitation it is possible to discriminate between X-bearing spermatozoa and Y-bearing spermatozoa by their differing emission levels.

In order to achieve separation and isolation of the appropriate sperm cells, the signals  
10 received by sensor (14) are fed to some type of sorter discrimination system (15) which very rapidly makes a differentiation decision and can differentially charge each drop (11) based upon whether it has decided that the desired sperm cell does or does not exist within that drop (11). In this manner the separation or discrimination system (15) acts to permit the electrostatic deflection plates (16) to deflect drops (11) based on whether or not they contain  
15 the appropriate sperm cell. As a result, the flow cytometer acts to sort cells by causing them to land in one or more collectors or containment elements (17). Thus by sensing some property of the sperm cells (such as magnitude of fluorescence), the flow cytometer can discriminate between sperm cells based on that particular characteristic and place them in the appropriate collector or containment element (17). In particular embodiments of the  
20 invention using flow cytometry to sort spermatozoa, the X-bearing sperm cell containing droplets are charged positively and thus deflect in one direction, and the Y-bearing sperm cell containing droplets are charged negatively and thus deflect the other way, and the wasted stream (containing unsortable sperm cells) remain uncharged and thus can be collected in an undeflected stream into a suction tube, or the like.

25

Now referring primarily to Figure 3, the nozzle (4) emits a stream (10) which because of the oscillator (8) (not shown in Figure 3) forms drops (11). Since the sperm cell source (3) (not shown in Figure 3) may supply sperm cells (1) which may be stained according to the above-described invention, the light emission from the bound fluorochrome excited by laser  
30 exciter (13) can be differentially determined by sensor (14) so that the existence or nonexistence of a charge on each drop (11) as it separates from stream (10) can be controlled

by the flow cytometer. This control results in positively charged, negatively charged, or uncharged drops (8) based upon the sperm cell contained within each drop (11). As shown by Figure 3, certain drops are shown as deflected drops (18). These deflected drops (18) are those containing spermatozoon differentiated by bearing either an X-chromosome or a Y-chromosome. Separated spermatozoa are then isolated in an appropriate collection element or containment element (17) for later use.

Embodiments of the invention can comprise droplets (11) each containing a sperm cell (15) bearing either an X-chromosome or a Y-chromosome. Droplets containing X-chromosome bearing sperm cells can be isolated into containment element(s) (17) at a rate of at least 1000 per second or at a rate greater than about 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or higher. Similarly Y-chromosome bearing sperm cells can be isolated at a rate of at least 1000 per second or at a rate greater than about 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or higher. In some embodiments of the invention, droplets containing X-chromosome bearing sperm cells and droplets containing Y-chromosome bearing sperm cells are simultaneously separated and isolated into containment elements each at a rate of at least 1000 per second, or greater than 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or at even higher rates.

20

Embodiments of the invention can also include artificial insemination samples prepared from sperm cells collected from male mammals (which can be frozen and thawed with respect to some embodiments of the invention) that are then stained and separated according to embodiments of the invention above-described. The artificial insemination samples can then be utilized in artificial insemination protocols. For example, a bovine artificial insemination sample prepared from separated spermatozoa according to the invention can comprise fewer than  $10 \times 10^6$  viable spermatozoa contained within a straw. Low dose artificial insemination samples for bovine artificial insemination can contain as few as  $1-3 \times 10^6$  viable spermatozoa, or even as few as 150,000 spermatozoa as described in United States Patent Application 09/001,394, or PCT Patent Application US98/27909, each hereby incorporated by reference. Artificial insemination samples, having a regular number

30

of separated sperm cells or a low dose of separated sperm cells can be used in animal breeding programs, such as those described in United States Patent Applications 60/224,050 and 60/21,093, each hereby incorporated by reference. Artificial insemination samples containing previously frozen and thawed spermatozoa stained and separated according to the invention can also be utilized in conjunction with synchronized breeding programs using superovulated animals as described in United States patent Application 09/001,454, hereby incorporated by reference herein. Naturally, for frozen sperm cells that are of limited availability because the male mammal is deceased, or the male mammal is a rare or exotic animal, an artificial insemination sample prepared according to the invention may contain even fewer spermatozoa.

The number of viable separated spermatozoa that are stained, separated, and isolated into X-chromosome bearing or Y-chromosome bearing populations according to the invention that are used in an artificial insemination sample can vary based upon the species of mammal to be artificially inseminated. For example, equine artificial insemination samples prepared from separated spermatozoa may require a higher number of viable separated spermatozoa relative to the bovine application, as described in PCT Patent Application US99/17165, hereby incorporated by reference. An embodiment of an equine insemination sample may, as but one example, contain between about forty million to about one-hundred million spermatozoa.

In certain embodiments of the invention, the insemination sample containing separated spermatozoa collected from a male mammal or obtained from frozen-thawed sperm may be packaged for use with surgical insemination procedures

Sperm cells stained, separated, or isolated according to the invention can also be used to fertilize oocyte(s) in-vitro (IVF). An attractive feature of IVF can be that fewer separated sperm are need than for artificial insemination. It may be desirable to use the fewest sperm possible, especially if the male mammal is deceased, rare, or exotic or if the spermatozoa are stained or separated in accordance with various embodiments of the invention. Also, commercial availability of sperm cells separated into X-chromosome bearing and Y-

chromosome bearing populations, especially when the male mammal is located a distance from the female mammal, or is exotic, rare, or has desirable attributes, will likely result in greatly expanded use of IVF in breeding programs. Certain embodiments of the invention can include devices and methodologies for the use of separated spermatozoa, including but not limited to frozen-thawed sperm cells, with respect to the in-vitro fertilization of oocytes, the in-vitro oocyte maturation, or the in-vitro culture of zygotes, such as those described in the journal article by Lu, K.H., Cran D.G., and Seidel, G. E., In-vitro Fertilization With Flow Cytometrically-Sorted Bovine Sperm, Theriogenology, 52, 1393-1405 (1999), hereby incorporated by reference.

Certain embodiments of the invention involving the production or generation of mammalian embryos can comprise collection of semen (1) from a male mammal or obtaining semen or spermatozoa (1) that are or have been previously frozen. According to embodiments of the invention described above, the semen is combined with Hoechst 33342 (2) stain to establish a concentration of between 40  $\mu\text{M}$  and 2500  $\mu\text{M}$ . The sperm cells are incubated with the Hoechst 33342 stain at a temperature between about 30 ° Centigrade and about 40° Centigrade for a duration of between about 50 minutes to about 200 minutes. The stained sperm cells may be separated and isolated into X-chromosome bearing and Y-chromosome bearing populations according to embodiments of the invention described above or by other sperm cell separation techniques that also differentiate X-chromosome bearing spermatozoa from Y-chromosome bearing spermatozoa based upon the magnitude of fluorescence. The isolated sperm cells may then be used to fertilize oocytes from a female mammal of the same species, and in some cases from female mammals of different species, in-vitro.

As an example of an application of embodiments of the invention involving frozen bull sperm in IVF applications, sperm samples from two bulls were stained either at a concentration of 224  $\mu\text{M}$  or 2,240  $\mu\text{M}$  of Hoechst 33342 and the stained spermatozoa were then bulk sorted on a flow cytometer at 1000 sperm/sec into 2% egg yolk citrate. Spermatozoa were inseminated at  $1 \times 10^6/\text{mL}$  and embryos were cultured in the mSOF system described by Tervit H.R. et al., Successful Culture In-Vitro of Sheep and Cattle Ova, J.



Reprod. Fertil., 30:493-497 (1992), hereby incorporated by reference. Three replicates were carried out for bull 1 and one replicate for bull 2 (Table 1).

Table 1. Effect of stain concentration on cleavage and developmental rates of oocytes inseminated with separated stained spermatozoa from frozen-thawed sperm.

Bull	No. Ejaculates	Hoechst 33342 conc. ( $\mu$ M)	Staining time required (min)	No. oocytes	% cleave	% blastocysts/oocyte
1	3	224	190	368	44 <sup>a</sup>	17
1	3	2240	60	373	60 <sup>b</sup>	23
2	1	224	190	86	23 <sup>a</sup>	0 <sup>a</sup>
2	1	2240	60	81	42 <sup>b</sup>	16 <sup>b</sup>

<sup>a,b</sup> Percentages within bulls within columns with different superscripts differ ( $P < .025$ ,  $\chi^2$ )

As can be understood, It can take much longer to stain frozen-thawed sperm so that they can be resolved during separation at the lower stain concentration than at 10X stain concentration. The differences observed in cleavage rates between the two stain concentrations most likely can be attributed to the extended incubation time at the lower stain level. It appears that a 10-fold increase in stain concentration does not have depressive effect on either cleavage or embryonic development.

15

As can be easily understood from the foregoing, the basic concepts of the present invention may be embodied in a variety of ways. It involves the staining of spermatozoa, whether fresh spermatozoa or frozen-thawed spermatozoa, separation and isolation techniques which may be used with such stained spermatozoa, as well as devices to accomplish the staining, separation, and isolation of such stained spermatozoa into X-chromosome bearing and Y-chromosome bearing populations. In this patent application, the staining and separating techniques used with spermatozoa are disclosed as part of the results

shown to be achieved by the various devices described and as steps which are inherent to utilization. They are simply the natural result of utilizing the devices as intended and described. In addition, while some devices are disclosed, it should be understood that these not only accomplish certain methods but also can be varied in a number of ways.  
5 Importantly, as to all of the foregoing, all of these facets should be understood to be encompassed by this disclosure.

The discussion included in this international Patent Cooperation Treaty patent application is intended to serve as a basic description. The reader should be aware that the  
10 specific discussion may not explicitly describe all embodiments possible; many alternatives are implicit. It also may not fully explain the generic nature of the invention and may not explicitly show how each feature or element can actually be representative of a broader function or of a great variety of alternative or equivalent elements. Again, these are implicitly included in this disclosure. Where the invention is described in functionally-  
15 oriented terminology, each aspect of the function is accomplished by a device, subroutine, or program. Apparatus claims may not only be included for the devices described, but also method or process claims may be included to address the functions the invention and each element performs. Neither the description nor the terminology is intended to limit the scope of the claims which now be included.

20

Further, each of the various elements of the invention and claims may also be achieved in a variety of manners. This disclosure should be understood to encompass each such variation, be it a variation of an embodiment of any apparatus embodiment, a method or process embodiment, or even merely a variation of any element of these. Particularly, it  
25 should be understood that as the disclosure relates to elements of the invention, the words for each element may be expressed by equivalent apparatus terms or method terms -- even if only the function or result is the same. Such equivalent, broader, or even more generic terms should be considered to be encompassed in the description of each element or action. Such terms can be substituted where desired to make explicit the implicitly broad coverage to  
30 which this invention is entitled. As but one example, it should be understood that all actions may be expressed as a means for taking that action or as an element which causes that action.

Similarly, each physical element disclosed should be understood to encompass a disclosure of the action which that physical element facilitates. Regarding this last aspect, as but one example, the disclosure of a "sorter" should be understood to encompass disclosure of the act of "sorting" -- whether explicitly discussed or not -- and, conversely, were there only disclosure of the act of "sorting", such a disclosure should be understood to encompass disclosure of a "sorter" and even a "means for sorting". Such changes and alternative terms are to be understood to be explicitly included in the description. Additionally, the various combinations and permutations of all elements or applications can be created and presented. All can be done to optimize the design or performance in a specific application.

10

Any acts of law, statutes, regulations, or rules mentioned in this application for patent: or patents, publications, or other references mentioned in this application for patent are hereby incorporated by reference. Specifically, United States Provisional Patent Application No. 60/253,787, filed November 29, 2000 and United States Provisional Patent Application No. 60/253,785, filed November 29, 2000, are hereby incorporated by reference including any figures or attachments, and each of references in the following table of references are hereby incorporated by reference.

15

#### US Patent Documents

DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS	FILING DATE
32,350	02/10/87	Bhattacharya			11/22/74
3,687,806	08/29/72	Van den Bovenkamp	195	1.3	11/04/69
3,829,216	08/13/74	Persidsky	356	36	10/02/72
3,894,529	07/15/75	Shrimpton	128	1 R	04/10/69
4,009,260	02/22/77	Ericsson	424	105	12/11/74
4,067,965	01/10/78	Bhattacharya	424	105	12/17/75
4,083,957	04/11/78	Lang	424	78	02/04/76
4,085,205	04/18/78	Hancock	424	105	01/24/77
4,092,229	05/30/78	Bhattacharya	204	180 R	10/20/76
4,155,831	05/22/79	Bhattacharya	207	299 R	02/23/78
4,191,749	03/04/80	Bryant	424	105	10/11/77
4,225,405	09/30/80	Lawson	204	180 R	08/16/78
4,276,139	06/30/81	Lawson	204	180 R	10/09/79
4,339,434	07/13/82	Ericsson	424	105	08/17/81
4,362,246	12/07/82	Adair	209	3.3	07/14/80
4,448,767	05/15/84	Bryant	424	85	02/15/80
4,474,875	10/02/84	Shrimpton	435	002	08/18/80
4,501,366	02/26/85	Thompson	209	556	12/14/82
4,511,661	04/16/85	Goldberg	436	503	12/30/83

4,605,558	08/12/86	Shrimpton	424	561	04/20/84
4,660,971	04/28/87	Sage et al.	356	39	05/03/84
4,680,258	07/14/87	Hammerling et al	435	7	08/09/83
4,673,288	06/16/87	Thomas et al.			
4,683,195	07/28/97	Mullis et al			
4,683,202	07/28/87	Mullis			
4,698,142	10/06/87	Muroi et al	204	182.3	07/31/85
4,749,458	06/07/88	Muroi et al	204	182.3	03/02/87
4,790,653	12/13/88	North, Jr.			
4,988,619	01/29/91	Pinkel	435	30	11/30/87
4,999,283	03/12/91	Zavos et al	435	2	08/18/89
5,021,244	06/04/91	Spaulding	424	561	05/12/89
5,055,393	10/08/91	Kwoh et al			
5,135,759	08/04/92	Johnson	424	561	04/26/91
5,346,990	09/13/94	Spaulding	530	350	03/12/91
5,371,585	12/06/94	Morgan et al.	356	246	11/10/92
5,437,987	08/01/95	Ten et al			
5,439,362	08/08/95	Spaulding	424	185.1	07/25/94
5,461,145	10/24/95	Kudo et al			
5,466,572	11/14/95	Sasaki et al.	435	2	04/25/94
5,480,774					
5,483,469	01/09/96	Van den Engh et al.	364	555	08/02/93
5,494,795	2/27/96	Guerry et al.	435	6	5/5/93
5,503,994	04/02/96	Shear et al.	436	90	10/08/93
5,578,449	11/26/96	Frasch et al.	435	6	4/20/95
5,514,537	05/07/96	Chandler	435	002	11/28/94
5,589,457	12/31/96	Wiltbank	514	12	07-03-95
5,602,039	02/11/97	Van den Engh	436	164	10/14/94
5,602,349	02/11/97	Van den Engh	73	864.85	10/14/94
5,622,820	4/11/97	Rossi	435	5	11/3/94
5,641,457	03/09/99	Tomiyama et al.	250	207	06/16/97
5,643,796	07/01/97	Van den Engh et al	436	50	10/14/4
5,660,997	08/26/97	Spaulding	435	7.21	06/07/95
5,690,895	11/25/97	Matsumoto et al.	422	73	12/06/96
5,700,692	12/23/97	Sweet	436	50	09/27/94
5,726,364	03/10/98	Van den Engh	73	864.85	02/10/97
5,819,948	10/13/98	Van den Engh	209	158	08/21/97
5,876,942	3/2/99	Cheng et al	435	6	7/24/97
5,880,457	03/09/99	Tomiyama et al.	250	207	06/16/97
5,985,216	11/16/99	Rens, et al.	422	073	07/24/97
6,071,689	06/06/00	Seidel et al.	435	2	01/29/98

## Foreign Patent Documents

DOCUMENT NO	DATE	COUNTRY
WO 96/12171	10/13/95	United States
WO 98/34094	06/08/98	NZ
WO 99/05504	07/24/98	US
WO 99/33956	08/07/99	US
WO 99/38883	05/08/99	US
WO 99/42810	08/26/99	US
WO 00/06193	10/02/00	US

## Other Reference Documents

Roser, J.F., Evans, J.W., Kiefer, D.P., Neeley, D.P. and Pacheco, C.A. 1980. Reproductive efficiency in mares with anti-hCG antibodies. Proc 9 <sup>th</sup> Int. Congr. Artira. Repro. and A.I. 4:627. abstr.
"Applying Semen Sexing Technology to the AI Industry", National Association of Animal Breeders, September 2000, pp. 1-16
"Sexed Semen Offers Faster Genetic Gain", Farming News, Livestock Supplement, February 1997, p. 28.
Akhtar, S., et al., "Prevalence of Five Stereotypes of Bluetongue Virus in a Rambouillet Sheep Flock in Pakistan", Veterinary Record 136, 1995, p. 495.
Akhtar, S., et al., "Sex Preselected in Cattle: a Field Trial", Veterinary Record 136, 1995, p. 495-496.
Aldrich, S. L., Berger, L.L., Reiling, B.A., Kegler, D.I., and Nagh, T.G.. 1995. "Parturition and periparturient reproductive and metabolic hormone concentration in prenatally androgenized beefheifer", I. Anim. Sci. 73:3712.
Amann, R. P. "Issues affecting commercialization of sexed sperm". Therio: 52:1441, 1999
Amann, R.P. et al, "Prospects For Sexing Mammalian Sperm," Colorado Associated University Press, Animal Reproduction Laboratory College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, 80523, 1982
American Meat Science Association in cooperation with National Livestock and Meat Board. "Research guidelines for cookery, sensory evaluation and instrumental tenderness measurements of fresh meat", 1995
Amoah, E.A. and Gelaye, S. 1996. Biotechnological advances in goat reproduction. J. Anim. Sci. 75(2):578-585.
Andersen, V.K., Aamdal, J. and Fougner, J.A. 1973. Intrauterine und tiefzervikale Insemination mit Gefriersperma beim Schaf. Zuchthygiene. 8:113-118.
Bagley, C. P. 1993. Nutritional management of replacement beef heifers -A review. J. Anim. Sci. 71:3155-3163.
Bailey, C. M., Reid, C.R., Ringkob, T.P., Koh, Y.O., and Foote, W.D. "Nulliparous versus primiparous crossbred females for beef." J. Anim. Sci. 69:1403., 1991
Baker, R.D., Dziuk, P.J. and Norton, H.W. 1968. Effect of volume of semen, number of sperm and drugs on transport of sperm in artificially inseminated gilts. J. Anim. Sci. 27:88-93.
Barnes, F.L. and Eyestone, W.H., "Early Cleavage and the Maternal Zygotic Transition in Bovine Embryos", Theriogenology, Vol. 33, No. 1, January 1990, pp. 141-149
Becker, S.E. and Johnson, A.L. 1992. Effects of gonadotropin releasing hormone infused in a pulsatile or continuous fashion on serum gonadotropin concentrations and ovulation in the mare. J. Anim. Sci. 70:1208-1215.
Bedford, S. J. and Hinrichs, K. 1994. The effect of insemination volume on pregnancy rates of pony mares. Theriogenology 42:571-578.
Bellows, R. A., Short, R.E., Anderson, D.C., Knapp, B.W., and Pahnish, O.F. "Cause and effect relationships associated with calving difficulty and calfbirth weight", J. Anim. Sci. 33:407, 1971
Berardinelli, J. G., R. A. Dailey, R. L. Butcher, and E. K. Inskeep. "Source of progesterone prior to puberty in beef heifers". J. Anim. Sci. 49:1276., 1979
Berger, G.S. 1987. Intratubal insemination. Fert. Steril. 48:328-330.
Bergfeld, E. G., Kojima, F.N., Cupp, A.S., Wehmann, M.E., Peters, K.T., Garcia-winder, M., and Kinder, J.E., "Ovarian follicular development in prepubertal heifers is influenced by level of dietary energy-intake", Bio. of Repro. 51:1051, 1994
Berry, B. W., Smith, G.C., and Carpenter, J., "Beef carcass maturity indicators and palatability attributes", J. Anim. Sci. 38:507, 1974
Beyhan, Z., et al., "Sexual Dimorphism in IVF Bovine Embryos Produced by Sperm Sorted by High Speed Flow Cytometry", Theriogenology 49, 1998, p. 359.
Blanchard, T. and Dickson, V., "Stallion Management", The Veterinary Clinics of North America, Equine Practice, Vol. 8, No. 1, April 1992, pp 207 - 218.
Bond, J., et al., "Growth and carcass traits of open beef heifers versus beef heifers that have calved", Nutrition Reports International 34:621. 1986
Boucq, C. V., et al., "Beef-production with maiden and once-calved heifers", Livestock Prod. Sci. 7:121. 1980
Bourdon, R. M. and J. S. Brinks. "Simulated efficiency of range beef -production". Culling strategies and nontraditional management-systems. J. Anim. Sci. 65:963. 1987
Bracher, V. and Allen, W.R., "Videoendoscopic Examination of the Mare's Uterus: Findings in Normal Fertile Mares", Equine Veterinary Journal, Vol. 24 (1992), pp. 274-278

Braselton, W.E. and McShan, W.H. 1970. "Purification and properties of follicle stimulating and luteinizing hormones from horse pituitary glands", Arch. Biochem. Biophys. 139:45-48.
Brethour, J. R., "The single-calfheifer system", Kans. Agric. Sta. Rep. Frog. 570. 1989
Bristol, S.P. 1982. Breeding behavior of a stallion at pasture with 20 mares in synchronized oestrus. J. Reprod. Fert. Suppl. 32:71.
Brookes, A. J. and Obyrne, M., "Use of cow-heifers in beef production", J. of the Royal Agricultural Society of England 126:30. 1965
Buchanan, B.R., et al, "Insemination of Mares with Low Numbers of Either Unsexed or Sexed Spermatozoa", Theriogenology, Vol. 53, pp 1333-1344, (2000)
Burns, P. D. and Spitzer, J.C., "Influence of biostimulation on reproduction in postpartum beef-cows", J. Anim. Sci. 70:358. 1992
Burwash, L.D., Pickett, B.W., Voss, J.L. and Back, D.G. 1974. "Relationship of duration of estrus to pregnancy rate in normally cycling, non-lactating mares" J.A.V.M.A. 165:714-716.
Byerley, D. J., et al., "Pregnancy rates of beef heifers bred either on puberal or 3rd estrus". J Anim. Sci. 65:645. 1987
Caslick, E.A., "The Vulva and the Vulvo-vaginal Orifice and its Relation to Genital Health of the Thoroughbred Mare", Cornell Veterinarian, Vol. 27, 1937, pp. 178-187
Catt, et al., "Assessment of Ram and Boar Spermatozoa During Cell-Sorting by Flow Cytometry", Reproduction Dom Animal, Vol. 32, 1997, pp 251-258.
Catt, S.L., et al., "Birth of a Male Lamb Derived from an In Vitro Matured Oocyte Fertilized by Intracytoplasmic Injection of a Single Presumptive Male Sperm", Veterinary Record 139, 1996, pp. 494-495.
Chin, W.W. and Boime, I. 1990. In: Glycoprotein Hormones. Serona Symp. Norwell, MA. pp. 19-20
Chung, Y.G., Schenk, J.L., Herickhoff, L.A. and Seidel, G.E. Jr. 1998. Artificial insemination of superovulated heifers with 600,000 sexed sperm. J Anim. Sci. Suppl. 1. 836:215. abstr.
Clement, F., Vincent, P., Mahla, R., Meriaux, J.C. and Palmer, E. 1998. Which insemination fertilizes when several successive inseminations are performed before ovulation. 7 <sup>th</sup> Int. Symp. Eq. Repro. 151. abstr.
Coleou, J., et al., "Essai de velage tres precoce de genisses en vue de la production de viande." Essai Vauz/ Aure no.50, programme USFGC-INAPG-ITFC. 1974
Cran, D.G., et al., "Production of Bovine Calves Following Separation of X- and Y- Chromosome Bearing Sperm and In Vitro Fertilisation", Veterinary Record 132, 1993, pp. 40-41.
Cran, D.G., et al., "Production of Lambs by Low Dose Intrauterine Insemination with Flow Cytometrically Sorted and Unsorted Semen", Theriogenology 47, 1997, p. 267.
Crowley, J. P. The facts of once-bred heifer production. (Ed) J.B. Owens. The maiden female-a means of increasing meat production. School of Agric., Univ. of Aberdeen, Scotland. 1973
Curran, S. 1998. In: Equine Diagnostic Ultrasonography. Fetal gender determination. Rantanen & McKinnon. 1 <sup>st</sup> Ed. Williams and Wilkins. pp. 165-169.
Day, B.N., Abeydeera, L.R., Johnson, L.A., Welch, G.R., Wang, W.H., Cantley, T.C. and Rieke, A. 1998. Birth of piglets preselected for gender following in vitro fertilization of in vitro matured pig oocytes by X and Y bearing spermatozoa sorted by high speed flow cytometry. Theriogenology. 49(1):360. abstr.
Dean, P.N., Pinkel, D. and Mendelsob. n, M.L. 1978. Hydrodynamic orientation of spermatozoa heads for flow cytometry. Biophys. J. 23:7-13.
Demick, D.S., Voss, J.L. and Pickett, B.W. 1976. Effect of cooling, storage, glycerization and spermatozoal numbers on equine fertility. J. Anim. Sci. 43:633-637.
DenDaas, J.H.G., De Jong, G., Lansbergen, L.M.T.E. and Van Wagendonk-De Leeuw, A.M. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of dairy bulls. J Dairy Sci. 81: 1714-1723.
Denham, A. "In-vitro studies on sandhill range forage as related to cattle preference", M.S. Thesis. 1965. Colorado State University.
Deutscher, G. H. "Extending interval from seventeen to nineteen days in the melengestrol acetate-prostaglandin estrous synchronization program for heifers". The Professional Animal Scientist 16:164. 2000
"Diagnostic Products Corporation. Coat-A-Count", Progesterone.com. 1998.
Dikeman, M. E. "Cattle production systems to meet future consumer demands. J. Anim. Sci. 59:1631, 1984
Dinnyes, A., et al., "Timing of the First Cleavage Post-insemination Affects Cryosurvival of In Vitro-produced Bovine Blastocysts", Molec Reprod Develop 53, 1999, pp 318-324.

Donaldson, L. E., "Effect of Insemination Regimen on Embryo Production in Superovulated Cows", The Veterinary Record, July 13, 1985, pp. 35-37
Donoghue, A.M., Byers, A.P., Johnston, L.A., Armstrong, D.L. and Wildt, D.E. 1996. Timing of ovulation after gonadotropin induction and its importance to successful intrauterine insemination in the tiger ( <i>Panthera tigris</i> ). J. Reprod. Fert. 107:53-58.
Douglas, R.H. 1979. Review of superovulation and embryo transfer in the equine. Theriogenology. 11:33-46.
Douglas, R.H., Nuti, L. and Ginther, O.J. 1974. Induction of ovulation and multiple ovulation on seasonally-anovulatory mares with equine pituitary fractions. Theriogenology. 2(6): 133-142.
Doyle, S. P., et al. "Artificial insemination of lactating angus cows with sexed semen". Proc. Western Sect. Am.Soc.Anim. Sci. 50:203. 1999
Duchamp, G., Bour, B., Combamous, Y. and Palmer, E. 1987. Alternative solutions to hCG induction of ovulation in the mare. J. Reprod. Fert. Suppl. 35:221-228.
Evans, M.J. and Irvine, C.H.G. 1977. Induction of follicular development, maturation and ovulation by gonadotropin releasing hormone administration to acyclic mares. Bio. Reprod. 16:452-462.
Ferrell, C. L. and T. G. Jenkins. "Energy-Utilization by Mature, nonpregnant, nonlactating cows of different types" J. Anim. Sci. 58:234. 1984
Ferrell, C. L. "Effects of post-weaning rate of gain on onset of puberty and productive performance of heifers of different breeds. J. Anim. Sci. 55:1272. 1982
Field, R. A., et al., "Bone-ossification and carcass characteristics of wethers given silastic implants containing estradiol". J. Anim. Sci. 68:3663-3668. 1990
Field, R., R. et al., "Growth, carcass, and tenderness characteristics of virgin, spayed, and single-calf heifers.", J. Anim. Sci. 74:2178. 1996
Fitzgerald, B.P., Peterson, K.D. and Silvia, P.J. 1993. Effect of constant administration of a gonadotropin-releasing hormone agonist on reproductive activity in mares: Preliminary evidence on suppression of ovulation during the breeding season. Am. J. Vet. Res. 54:1746-1751.
Fluharty, F. L., et al., "Effect of weaning and diet on growth of calves." Research and Reviews. The Ohio State University Department of Animal Sciences. 1996
Fluharty, F.L., et al., "Effects of Age at Weaning and Diet on Growth of Calves", Ohio Agri. Res. and Dev. Circular, 1996, 156: 29.
Foulkes, J.A., Stewart, D.L. and Herbert, C.N. 1977. Artificial insemination of cattle using varying numbers of spermatozoa. Vet. Rec. 101:205.
Fugger, E.F., "Clinical Experience with Flow Cytometric Separation of Human X- and Y- Chromosome Bearing Sperm", Theriogenology, Vol. 52, pp. 1435-1440 (1999)
Fulwyler, M.J. 1965. Electronic separation of biological cells by volume. Science. 150:910.
Fulwyler, M.J. 1977. Hydrodynamic orientation of cells. J Histochem. Cytochem. 25:781-783.
Seidel, G.E., Jr., "Artificial Insemination With X-and Y-Bearing Bovine Sperm", Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO; Germplasm and Gamete Physiology Lab, ARS, USDA, Beltsville, MD; Atlantic Breeders Coop, Lancaster, PA; DUO Dairy, Loveland, CO, USA January 1996.
Garner, D.L., Gledhill, B.L., Pinkel, D., Lake, S., Stephenson, D., Van Dilla, M.A. and Johnson, L.A. 1983. "Quantification of the X and Y chromosome-bearing spermatozoa of domestic animals by flow cytometry". Biol. Reprod. 28:312-321.
Ginther, O.J. 1983. Sexual behavior following introduction of a stallion into a group of mares. Theriogenology. 19:877.
Ginther, O.J. 1992. In: Reproductive Biology of the Mare. (2 <sup>nd</sup> Ed.) Equiservices, Cross Plains, WI.
Gledhill, B.L. 1988. Gender preselection: historical, technical and ethical perspective. Semin Reprod. Endocrinol. 6:385-395.
Gombe, S. and Hansel, W. "Plasma luteinizing-hormone (LH) and progesterone levels in heifers on restricted energy intakes." J. Anim. Sci. 37:728. 1973
Gourley, D.D. and Riese, R.L. 1990. Laparoscopic artificial insemination in sheep. Vet. Clin. N. Amer: Food Anim. Prac. 6(3):615-633.
Gravert, H. O., "Genetic Aspects of Early Calving." In: J.C. Taylor (Ed.) The early calving of heifers and its impact on beef production. 59. 1975
Gregory, K. E., et al., "Characterization of biological types of cattle III .2." Growth-rate and puberty in females. J.

Anim. Sci. 49:461. 1979
Grimes, I. F. and T. B. Turner. "Early weaning of fall born calves II." Post weaning performance of early and normal-weaned calves. I. Prod. Agric. 4:168. 1991
Grondahl, C., et al, "In Vitro Production of Equine Embryos", Biology of Reproduction, Monograph Series I, pp. 299-307 (1995)
Guillou, F. and Combarnous, Y. 1983. Purification of equine gonadotropins and comparative study of their acid-dissociation and receptor-binding specificity. Biochem. Biophys. Acta. 755:229-236.
Gurnsey, M.P., and Johnson, L.A., "Recent improvements in efficiency of flow cytometric sorting of X and Y-chromosome bearing sperm of domestic animals: a review", 1998, New Zealand Society of Animal Protection, three pages.
Hall, J. B., et al., "Effect of age and pattern of gain on induction of puberty with a progestin in beef heifers." J. Anim. Sci. 75:1606. 1997
Hamano, K., et al., "Gender Preselection in Cattle with Intracytoplasmically Injected, Flow Cytometrically Sorted Sperm Heads", biology of Reproduction 60, 1999, pp. 1194-1197.
Harrison, L.A., Squires, E.L. and McKinnon, A.O. 1991. Comparison of hCG, buserelin and luprostitol for induction of ovulation in cycling mares. Eq. Vet. Sci. 3:163-166.
Harte, F. J. "System of production of beef from once calved heifers." In: J.C. Taylor (Ed.) The early calving of heifers and its impact on beef production. 123. 1975
Hawk, H.W., et al., "Fertilization Rates in Superovulating Cows After Deposition of Semen on the Infundibulum Near the Uterotubal Junction or After Insemination with High Numbers of Sperm", XP-002103478, Theriogenology, May 1988, Vol. 29, No. 5, pp 1131-1142.
Hemlsmeyer, G. N., et al. "Effects of lactation and prenatal androgenization on the performance, carcass composition and longissimus muscle sensory characteristics of heifers in the single-calfheifer system." The Professional Animal Scientist 15:14. 1999
Hennegmeyer, G. N., et al. "Effects of prenatal androgenization and implantation on the performance and carcass composition of lactating heifers in the single-calfheifer system." The Professional Animal Scientist 15:173. 1999
Hilton, G. G., et al., "An evaluation of current and alternative systems for quality grading carcasses of mature slaughter cows." I. Anim. Sci. 76:2094. 1998
Ho, L., et al., "Influence of gender, breed and age on maturity characteristics of sheep." J. Anim. Sci. 67:2460-2470. 1989
Hofferer, S., Lecompte, F., Magallon, T., Palmer, E. and Combarnous, Y. 1993. Induction of ovulation and superovulation in mares using equine LH and FSH separated by hydrophobic interaction chromatography. J. Reprod. Fert. 98:597-602.
Hohenboken, W. D. "Applications of sexed semen in cattle production." Therio.52:1421. 1999
Holtan, D.W., Douglas, R.H. and Ginther, O.J. 1977. Estrus, ovulation and conception following synchronization with progesterone, prostaglandin F2 ct and human chorionic gonadotropin in pony mares. J. Anim. Sci. 44:431-437.
Householder, D.D., Pickett, B.W., Voss, J.L. and Olar, T.T. 1981. Effect of extender, number of spermatozoa and hCG on equine fertility. J. Equine Vet. Sci. 1:9-13.
Howard, J.G., Bush, M., Morton, C., Morton, F., Wentzel, K. and Wildt, D.E. 1991. Comparative semen cryopreservation in ferrets ( <i>Mustela putorius furo</i> ) and pregnancies after laparoscopic intrauterine insemination with frozen-thawed spermatozoa. J. Reprod. Fert. 92:109-118.
Howard, J.G., Roth, T.L., Byers, A.P., Swanson, W.F. and Wildt, D.E. 1997. Sensitivity to exogenous gonadotropins for ovulation and laparoscopic artificial insemination in the cheetah and clouded leopard. Biol. Reprod. 56:1059-1068.
Hunter, R.H.F. 1980. Transport and storage of spermatozoa in the female reproductive tract. Proc 4 <sup>th</sup> Int. Congr. Artira. Repro. and A.I. 9:227-233.
Hyland, J.H., Ainsworth, C.G.V. and Langsford, D.A. 1988. Gonadotropin-releasing hormone (GnRH) delivered by continuous infusion induces fertile estrus in mares during seasonal anovulation. Proc. Amer. Assoc. Eq. Prac. 181-190.
Irvine, C.H.G. and Alexander, S.L. 1993. In: Equine Reproduction. Edited by McKinnon and Voss. Lea and Febiger. Philadelphia, London. pp. 37.
Jafar, et al., "Sex Selection in Mammals: A Review", Theriogenology, vol. 46, 1996, pp 191-200.
Jarriage, R. "Age of cows at first calving in France." J.C. Taylor (Ed.) The early calving of heifers and its impact



on beef production. 10. 1975
Jasko, D.J., Martin, J.M. and Squires, E.L. 1992. Effect of volume and concentration of spermatozoa on embryo recovery in mares. <i>Theriogenology</i> . 37:1233-1239
Johnson L.A., et al., 1987. Flow cytometry of X- and Y- chromosome bearing sperm for DNA using an improved preparation method and staining with Hoechst 333-42. <i>Garnete Research</i> 17: 203-212
Johnson, "Gender preselection in Mammals: An overview", <i>Dtsch. Tierarztl. Wschr.</i> , Vol. 103, Aug./Sep. 1996, pp 288-291.
Johnson, A.L. 1986. Pulsatile release of gonadotropin releasing hormone advances ovulation in cycling mares. <i>Biol. Reprod.</i> 35:1123E 1130.
Johnson, A.L. and Becker, S.E. 1988. Use of gonadotropin-releasing hormone (GnRH) treatment to induce multiple ovulations in the anestrous mare. <i>Eq. Vet. Sci.</i> 8:130-134.
Johnson, L., "Sex Preselection by Flow Cytometric Separation of X and Y Chromosome-Bearing Sperm Based on DNA Difference: a Review", <i>Reproduction and Fertilization Development</i> 7, 1995, pp. 893-903.
Johnson, L., "Successful Gender Preselection in Farm Animals", <i>Agricultural Biotechnology</i> , 1998, pp. 439-452.
Johnson, L.A. 1988. Flow cytometric determination of spermatozoa sex ratio in semen purportedly enriched for X or Y bearing spermatozoa. <i>Theriogenology</i> . 29:265. abstr.
Johnson, L.A. 1992. Gender preselection in domestic animals using flow cytometrically sorted sperm. <i>J Anim. Sci. Suppl</i> 1.70:8-18.
Johnson, L.A. 1994. Isolation of X- and Y-bearing spermatozoa for sex preselection. <i>In: Oxford Reviews of Reproductive Biology</i> . Ed. HH Charlton. Oxford University Press. 303-326.
Johnson, L.A. 1995. Sex preselection by flow cytometric separation of X and Y chromosome bearing spermatozoa based on DNA difference: a review. <i>Reprod. Fert. Dev.</i> 7:893-903.
Johnson, L.A. and Schulman, J.D. 1994. The safety of sperm selection by flow cytometry. <i>Ham. Reprod.</i> 9(5):758.
Johnson, L.A., "Sex preselection in swine: altered sex ratios in offspring following surgical insemination of flow-sorted X- and Y-bearing sperm", <i>Reprod. Domest. Anim.</i> 26:309-314, 1991
Johnson, L.A., and Pinkel, D., "Modification of a Laser-Based flow Cytometer for High-Resolution DNA Analysis of Mammalian Spermatozoa", <i>Cytometry</i> 7, 1986, pp 268 - 273.
Johnson, L.A., et al., "Sex Preselection in Rabbits: Live Births from X and Y Sperm Separated by DNA and Cell Sorting", <i>Exceptional Paper-Rapid Publication</i> , XP-002103476, <i>Biology of Reproduction</i> 41, 199-203, 1989, pp 199-203.
Johnson, L.A., et al., 1994. Improved flow sorting resolution of X- and Y- chromosome bearing viable sperm separation using dual staining and dead cell gating. <i>Cytometry</i> 17 (suppl 7):83.
Johnson, L.A., Flook, J.P., Look, M.V. and Pinkel, D. 1987b. Flow sorting of X and Y chromosome bearing spermatozoa into two populations. <i>Gam. Res.</i> 16:203-212.
Johnson, L.A., Welch, G.R., Rens, W. and Dobrinsky, J.R. 1998. Enhanced flow cytometric sorting of mammalian X and Y sperm: high speed sorting and orienting no77.1e for artificial insemination. <i>Theriogenology</i> . 49(1):361. abstr.
Joseph, R. L. "Carcass composition and meat quality in once calved heifers." <i>In: J.C. Taylor (Ed.) The early calving of heifers and its impact on beef production.</i> 143. 1975
Joseph, R. L. and J. P. Crowley. "Meat quality of once-calved heifers." <i>Irish J. of Agric. Research</i> 10:281. 1971
Kachel, V., et al., A Uniform Lateral Orientation, Cused by Flow Forces, of Flat Particles in Flow-Through Systems@, <i>The Journal of Histochemistry and Cytochemistry</i> , 1997, Vol. 25, No. 7, pp 774 -780.
Kanayama, K., Sankai, T., Nariaik, K., Endo, T. and Sakuma, Y. 1992b. Pregnancy by means of tubal insemination and subsequent spontaneous pregnancy in rabbits. <i>J. Int. Med. Res.</i> 20:401-405.
Karabinus, et al., "Effects of Egg Yolk-Citrate and Milk Extenders on Chromatin Structured Viability of Cryopreserved Bull Sperm", <i>Journal of Dairy Science</i> , Vol. 74, No. 11, 1999, pp 3836-3848.
Keeling, P. C. B. M. S. T. G. D. I. a. P. W. J., "A modeling study of once-bred heifer beef production." <i>Proceedings of the New Zealand Society of Animal Production.</i> 51. 1991
Kilicarslan, M.R., Horoz, H., Senunver, S.C., Konuk, S.C., Tek, C. and Carioglu, B. 1996. Effect of GnRH and hCG on ovulation and pregnancy in mares. <i>Vet. Rec.</i> 139:119-120.
Kinder, J. E., et al. "Endocrine basis for puberty in heifers and ewes." <i>J. Repro. and Fertility</i> 393. 1995
Klindt, J. and J. D. Crouse. "Effect of ovariectomy and ovariectomy with ovarian auto transplantation on feedlot performance and carcass characteristics of heifers." <i>J. Anim. Sci.</i> 68:3481. 1990

Klosterman, E. W. and C. F. Parker. "Effect of size, breed and sex upon feed efficiency in beef cattle." North Central Regional Research Publication 235, Ohio Agric. Research and Development Center 1090:3. 1976
Kniffen, D. M., Wagner, W.R., and Lewis, P.E. "Effects of long-term estrogen implants in beef heifers." I. Anim. Sci. 77:2886. 1999
Koch, R. M., et al., "Characterization of biological types of cattle -Cycle-II .3." Carcass composition, quality and palatability. I. Anim. Sci. 49:448. 1919
Lapin, D.R. and Ginther, O.J. 1977. Induction of ovulation and multiple ovulations in seasonally anovulatory and ovulatory mares with an equine pituitary extract. J. Anim. Sci. 44:834-842.
Laster, D. B., "Factors affecting dystocia and effects of dystocia on subsequent reproduction in beef-cattle." J. Anim. Sci. 36:695. 1973
Lawrenz, R. 1985. Preliminary results of non-surgical intrauterine insemination of sheep with thawed frozen semen. J S Afr. Vet. Assoc. 56(2):61-63.
Levinson, G., et al, 1995. DNA-based X-enriched sperm separation as an adjunct to preimplantation genetic testing for the preparation of X-linked disease. Mol. Human Reprod. 10:979-982.
Lindsey, A., et al., "AHysteroscopic Insemination of Mares with Nonfrozen Low-dose Unsexed or Sex-sorted Spermatozoa", currently unpublished, pp. 1-15.
Linge, F. 1972. Faltforsok med djupfrost sperma (field trials with frozen sperm). Farskotsel. 52:12-13.
Loneragan, P., et al., "Effect of Time Interval from Insemination to First Cleavage on the Development of Bovine Embryos In Vitro and In Vivo", Theriogenology, 1999, p. 326
Long, C.R., Rath, D., Welch, G.R., Schreier, L.L., Dobrinsky, J.R. and Johnson, L.A. 1998. An vitro production of porcine embryos from semen sorted for sex with a high speed cell sorter: comparison of two fertilization media. @, Theriogenology. 49(1):363. abstr.
Loy, R.G. and Hughes, J.P. 1965. The effects of human chorionic gonadotropin on ovulation, length of estrus, and fertility in the mare. Cornell Vet. 56:41-50.
Lu, K.H., et al., "In Vitro Fertilization with Flow-Cytometrically-Sorted Bovine Sperm", Theriogenology 52, 1999, pp. 1393-1405.
Lynch, I. M., et al., "Influence of timing of gain on growth and reproductive performance of beef replacement heifers." I. Anim. Sci. 75:1715. 1997
Macmillan, K.L. and A.M. Day, "Prostaglandin F2a - A Fertility Drug In Dairy Cattle?", Ruakura Animal Research Station, Private Bag, Hamilton, New Zealand, Theriogenology, September 1982, Vol. 18 No. 3, pages 245-253
Martin, A. H., et al., "Characteristics of youthful beef carcasses in relation to weight, age and sex .3. meat quality attributes." Canadian I. Anim. Sci. 51:305. 1971
Martin, L. C., J. S. Brinks, R. M. Bourdon, and L. V. Cundiff. "Genetic-effects on beef heifer puberty and subsequent reproduction." J. Anim. Sci. 70:4006. 1992
Matsuda, Y. and Tobari, I. 1988. Chromosomal analysis in mouse eggs fertilized <i>in vitro</i> with sperm exposed to ultraviolet light (UV) and methyl and ethyl methanesulfonate (MMS and EMS). Mutat. Res. 198:131-144.
Matulis, R. J., F. K. McKeith, D. B. Faulkner, L. L. Berger, and P. George. "Growth and carcass characteristics of cull cows after different times-on-feed." J. Anim. Sci. 65:669. 1987
Mauleon, P. "Recent research related to the physiology of puberty." Commission of the European Communities. The early calving of heifers and its impact on beef production. 1975
Maxwell, W. and Johnson, L., "Chlortetracycline Analysis of Boar Spermatozoa after Incubation, Flow Cytometric Sorting, Cooling, or Cryopreservation", Molecular Reproduction and Development 46, 1997, pp. 408-418.
Maxwell, W.M.C., Evans, G., Rhodes, S.L., Hillard, M.A. and Bindon, B.M. 1993. Fertility of Superovulated Ewes after Intrauterine or Oviductal Insemination with Low Numbers of Fresh or Frozen-Thawed Spermatozoa. Reprod. Fertil. Dev. 5:57-63.
Mccormick, R. J. "The flexibility of the collagen compartment of muscle." Meat Sci. 36:79. 1994
McCue, P.M. 1996. Superovulation. Vet. Clin. N. Amer. Eq. Prac. 12:1-11.
McCue, P.M., Fleury, J.J., Denniston, D.J., Graham, J.K. and Squires, E.L. 1997. Oviductal insemination in the mare. 7 <sup>th</sup> Int Symp. Eq. Reprod. 133. abstr.
McDonald, L.E. 1988. Hormones of the pituitary gland. In: Veterinary Pharmacology and Therapeutics. 6 <sup>th</sup> ed. Edited by N.H. Booth and L.E. McDonald. Ames, Iowa State Univ. Press. pp. 590.
McKenna, T., Lenz, R.W., Fenton, S.E. and Ax, R.L. 1990. Nonreturn rates of dairy cattle following uterine body

or comual insemination. J. Dairy Sci. 73:1179-1783.
McKinnin, A. and Voss, J., "Equine Reproduction", Lea & Febiger, Philadelphia, 1993, pp 291, 299 - 302, 345 - 348, 739 - 797.
McKinnon, A. et al, 1993. Predictable ovulation in mares treated with an implant of the GnRH analogue deslorelin. Eq. Vet. J. 25:321-323.
McKinnon, A.O. et al, 1996. Repeated use of a GnRH analogue deslorelin (Ovuplant) for hastening ovulation in the transitional mare. Eq. Vet. J. 29:153-155.
McNutt, et al., "Flow Cytometric Sorting of Sperm: Influence on Fertilization and Embryo/Fetal Development in the Rabbits", Molecular Reproduction and Development, Vol. 43, 1996, pp 261-267.
Meilgaard, M., G. V. Civile, and B. T. Carr. "Sensor Evaluation Techniques." CRC Press Inc., Boca Raton, FL. 1991
Meinert, C., et al., "Advancing the time of ovulation in the mare with a short-term implant releasing the GnRH analogue deslorelin", Equine Veterinary Journal, 25, 1993, pp 65 - 68.
Merton, J., et al., "Effect of Flow Cytometrically Sorted Frozen/Thawed Semen on Success Rate of In Vitro Bovine Embryo Production", Theriogenology 47, 1997, pp. 295.
Meyers, P.J., Bowman, T., Blodgett, G., Conboy, H.S., Gimenez, T., Reid, M.P., Taylor, B.C., Thayer, J., Jochle, W. and Trigg, T.E. 1997. Use of the GnRH analogue, deslorelin acetate, in a slow release implant to accelerate ovulation in oestrous mares. Vet. Rec. 140:249-252.
Michaels, Charles, "Beef A.I. Facilities that work", Proc. Fifth N.A.A.B Tech. Conf. A.I. Reprod. Columbia, MO. pp. 20-22.
Michel, T.H., Rossdale, P.D. and Cash, R.S.G. 1986. Efficacy of human chorionic gonadotrophin and gonadatrophin releasing hormone for hastening ovulation in Thoroughbred mares. Eq. Vet. J. 6:438-442.
Miller, S.J. 1986. <i>Artificial Breeding Techniques in Sheep</i> . In Morrow, D.A. (ed): Current Therapy in Theriogenology 2. Philadelphia, WB Saunders.
Mirskaja, L.M. and Petrapavlovskii, V.V. 1937. The reproduction of normal duration of heat in the mare by the administration of Prolan. Probl. Zivotn. Anim. Breed. Abstr. 5:387.
Moe, P. W., H. F. Tyrrell, and W. P. Flatt. "Energetics of body tissue mobilization." J. of Dairy Sci. 54:548.
Molinia, F.C., Gibson, R.J., Brown, A.M., Glazier, A.M. and Rodger, J.C. 1998. Successful fertilization after superovulation and laparoscopic intrauterine insemination of the brushtail possum, <i>Trichosurus vulpecula</i> , and tammar wallaby, <i>Macropus eugenii</i> . J.Reprod. Fert. 112:9-17.
Moms, S. T., et al., "Biological efficiency: How relevant is this concept to beef cows in a mixed livestock seasonal pasture supply context?" Proceedings of the New Zealand Society of Animal Production 54:333. 1994
Monensin." J. Anim. Sci. 55:357-362. 1982
Moran, C., J. F. Quirke, and J. F. Roche. "Puberty in heifers -a review." Animal Reproduction Sci. 18:167. 1989
Morcom, C.B. and Dukelow, W.R. 1980. A research technique for the oviductal insemination of pigs using laparoscopy. Lab. Anim. Sci. 1030-1031.
Morgan, J. B., et al., "National beef tenderness survey." J. Anim. Sci.69:3274. 1991
Morris, L.H., et al., "Hysteroscopic insemination of small numbers of spermatozoa at the uterotubal junction of preovulatory mares", Journal of Reproduction and Fertility, Vol. 118, pp. 95-100 (2000)
Moseley, W. M., et al., 1982. "Relationship of Growth and Puberty in Beef Heifers Fed
Mount, D. E. "Fibrous and non-fibrous carbohydrate supplementation to ruminants grazing forage from small grain crops." M.S. Thesis. Colorado State University. 2000
Muller, W. and Gautier, F. 1975. Interactions of heteroaromatic compounds with nucleic acids. Euro. J Biochem. 54:358.
Munne, S. 1994. Flow cytometry separation of X and Y spermatozoa could be detrimental to human embryos. Hum. Reprod. 9(5):758
Myers, S. E., "Performance and carcass traits of early-weaned steers receiving either a pasture growing period or a finishing diet at weaning." J. Anim. Sci. 77:311. 1999
Myers, S. E., et al., "Comparison of three weaning ages on cow-calf performance and steer carcass traits." J. Anim. Sci. 77:323. 1999
Myers, S. E., et al., "Production systems comparing early weaning to normal weaning with or without creep feeding for beef steers." J. Anim. Sci. 77:300. 1999
Nix, I. P., I. C. Spitzer, and P. I. Chenoweth. "Serum testosterone concentration, efficiency of estrus detection and libido expression in androgenized beef cows." Therio. 49: 1195. 1998

Nowshari, et al., "Superovulation of Goats with Purified pFSH Supplemented with Defined Amounts of pLH", <i>Theriogenology</i> , Vol 43, 1995, pp 797-802.
Nowshari, et al., <i>Theriogenology</i> , Vol 43, 1995, pp 797-802.
NRC. Nutrient requirements for beef cattle. National Academy of Sci. National Research Council, Washington, DC. 1996
Olson, S.E. and Seidel, G.E. Jr., "Reduced Oxygen Tension and EDTA improve Bovine Zygote Development in a Chemically Defined Medium", <i>Journal of Animal Science</i> 78, 2000, pp. 152-157.
Owen, J. B. "The maiden female-a means of increasing meat production." <i>Proc. Symp. on the use of once bred heifers and gilts</i> . 1973
Pace, M.M. and Sullivan, J.J. 1975. Effect of timing of insemination, numbers of spermatozoa and extender components on pregnancy rates in mares inseminated with frozen stallion semen. <i>J Reprod. Fert. Suppl.</i> 23:115-121.
Parent US Application 09/001,394, entitled "Sheath Fluids and Collection Systems for Sex-Specific Cytometer Sorting of Sperm", filed on December 31, 1997, 87 total pages which includes four drawings.
Parrish, J., et al., "Capacitation of Bovine Sperm by Heparin", <i>Technology of Reproduction</i> 38, 1988, pp. 1171-1180.
PCT application, PCT/US99/17165, filed 28 July 1999, entitled "Equine System for Non-Surgical Artificial Insemination".
PCT application, PCT/US98/27909, filed 31 December 1998, entitled "Commercially Practical Sex-Specific Insemination of Mammals".
Peippo, J., et al., "Sex diagnosis of equine preimplantation embryos using the polymerase chain reaction", <i>Theriogenology</i> , Vol. 44 619-627 (1995)
Perry, E.J. 1968. Historical Background In: <i>The Artificial Insemination of Farm Animals</i> . 4 <sup>th</sup> ed. Edited by E.J. Perry. New Brunswick, Rutgers University Press, pp. 3-12.
Petersen, G.A., et al, "Cow and Calf Performance and Economic Considerations of Early Weaning of Fall-Born Beef Calves", <i>J. Anim. Sci.</i> , 1987, 64:15, pp 15-22.
Petit, M. "Early Calving in Suckling Herds." In: (Ed.) J.C. Taylor. <i>The early calving of heifers and it's impact on beef production</i> . 157. 1975
Pickett GW, et al., "Management of the mare for maximum reproductive efficiency", <i>Bulletin No. 6 Colorado State University</i> , Ft. Collins CO. (1989)
Pickett, B.W, et al., 1976. Factors influencing the fertility of stallion spermatozoa in an A.I. program. <i>Proc. 8<sup>th</sup> Internat. Congr. Anim. Reprod. A.I. Krakow, Poland</i> . 4: 1049 - 1052.
Pickett, B.W. and Back, D.G. 1973. Procedures for preparation, collection, evaluation and insemination of stallion semen. <i>C.S.U. Exp. Sta. Artira. Reprod. Lab. Gen. Series Bull.</i> 935.
Pickett, B.W., and Shiner, K.A., "Recent developments in artificial insemination in horses", <i>Livestock Production Science</i> , 40, 1994, pp 31 - 36.
Pickett, B.W., Burwash, L.D., Voss, J.L. and Back, D.G. 1975b. Effect of seminal extenders on equine fertility. <i>J. Anim. Sci.</i> 40:1136-1143.
Pinkel, D., et al, "Flow Cytometric Determination of the Proportions of X- and Y- Chromosome-Bearing Sperm in Samples of Purportedly Separated Bull Sperm", <i>Journal of Animal Science</i> , Vol. 60, No. 5, 1985, pp 1303 - 1307.
Pinkel, D., Gledhill, B.L., Van Dilla, M.A., Stephenson, D. and Watchmaker, G. 1982b. High resolution DNA measurements of mammalian spermatozoa. <i>Cytometry</i> . 3:1-9. (1982b)
Polge, E. J., "Historical Perspective of AI: Commercial Methods of Producing Sex Specific Semen, IVF Procedures", <i>Proceedings of the 16<sup>th</sup> Technical Conference on Artificial Insemination &amp; Reproduction</i> , Cambridge, England, 1996, pp. 7-11.
Purvis, H. T. and J. C. Whittier. "Effects of ionophore feeding and anthelmintic administration on age and weight at puberty in spring-born beef heifers." <i>J. Anim. Sci.</i> 74:736-744. 1996
Randel, R. D. "Nutrition and postpartum rebreeding in cattle." <i>J. Anim. Sci.</i> 68:853. 1990
Rath, D., et al., "Low Dose Insemination Technique in the Pig", <i>Boar Semen Preservation IV</i> , 2000, pp. 115-118.
Rath, D., et al., "Production of Piglets Preselected for Sex Following in Vitro Fertilization with X and Y Chromosome-Bearing Spermatozoa Sorted by Flow Cytometry", <i>Theriogenology</i> , 47, 1997, pp 795 - 800.
Reiling, B.A., et al., "Effect of Prenatal Androgenization on Performance, Location, and Carcass and Sensory Traits on Heifers in Single Calf Heifer System", <i>J. Anim. Sci.</i> , 1995, 73: 986, pp 986-992.

Rens, W., et al, "A Novel Nozzle for More Efficient Sperm Orientation to Improve Sorting Efficiency of X and Y Chromosome-Bearing Sperm", <i>Cytometry</i> 33, 1998, pp. 476-481
Rens, W., et al., "Improved Flow Cytometric Sorting of X- and Y- Chromosome Bearing Sperm: Substantial Increase in Yield of Sexed Semen", <i>Molecular Reproduction and Development</i> , 1999, pp 50-56.
Rieger, D., et al, "The Relationship Between the Time of First Cleavage of Fertilized Cattle Oocytes and Their Development to the Blastocyst Stage", <i>Theriogenology</i> , 1999, pp. 190.
Ritar, A. and Ball, A. 1991. Fertility of young cashmere goats after laparoscopic insemination. <i>J. Agr. Sci.</i> 117:271-273.
Roberts, J.R. 1971. In: <i>Veterinary Obstetrics and Genital Diseases</i> . Ithaca, New York. pp. 740-749.
Romita, A. "Some considerations on the beef situation in Italy." (Ed.) J.C. Taylor. <i>The early calving of heifers and it's impact on beef production</i> . 23. 1975
Roth, T.L., Wolfe, B.A., Long, J.A., Howard, J. and Wildt, D.E. 1997. Effects of equine chorionic gonadotropin, human chorionic gonadotropin, and laparoscopic artificial insemination on embryo, endocrine, and luteal characteristics in the domestic cat. <i>Bio Reprod.</i> 57:165-171.
Roux, M., J. H. Teissier, J. Bonnemare, and R. Dumont. "Early calving heifers versus maiden heifers for beef -production from dairy herds. 1." The effects of genotype (Friesian and Charolais x Friesian) and 2 feeding levels in the rearing period on growth and carcass quality. <i>Livestock Prod. Sci.</i> 16:1. 1987
Rowley, H-S., Squires, E.L. and Pickett, B.W. 1990. Effect of insemination volume on embryo recover}' in mares. <i>J. Equine Vet. Sci.</i> 10:298-300.
Roy, J. H. B. "Rearing dairy-herd replacements." <i>J. of the Soc. of Dairy Technology</i> 31:73-79. 1978
Rutter, L. M., et al., "Effect of abomasal infusion of propionate on the GnRH-induced luteinizing-hormone release in prepubertal heifers." <i>J. Anim. Sci.</i> 56:1167. 1983
Salamon, S. 1976. <i>Artificial Insemination of Sheep</i> . Chippendale, New South Wales. Publicity Press. p.83-84.
Salisbury, G.W. and VanDemark, N.L. 1961. <i>Physiology of Reproduction and Artificial Insemination of Cattle</i> . San Francisco: Freeman and Company.
SAS, SAS/STAT, "User's Guide (Release 6.03)", SAS Inst. Inc., Cary, NC., 1988. 3 pages
SAS. "The SAS System for Windows." Ver 7.0. Rel 6.12. SAS Inst. Inc., Cary, NC. 2000
Schenk, J. L., T. K. Suh, D. G. Cran, and G. E. Seidel. "Cryopreservation of flow-sorted bovine spermatozoa." <i>Therio.</i> 52:1375. 1999
Schenk, J.L. and Seidel, Jr., G.E., "Imminent Commercialization of Sexed Bovine", <i>Proceedings, The Range Beef Cow Symposium XVI</i> , 1999, pp 89-96.
Schillo, K. K., J. B. Hall, and S. M. Hileman. "Effects of nutrition and season on the onset of puberty in the beef heifer." <i>J. Anim. Sci.</i> 70:3994. 1992
Schmid R.L., et al, "Fertilization with Sexed Equine Spermatozoa Using Intracytoplasmic Sperm Injection and Oviductal Insemination", 7th International Symposium On Equine Reproduction, pp. 139 (Abstract) (1998)
Schnell, T. D., K. E. Belk, J. D. Tatum, R. K. Miller, and G. C. Smith. "Performance, carcass, and palatability traits for cull cows fed high-energy concentrate diets for 0, 14, 28, 42, or 56 days." <i>J. Anim. Sci.</i> 75:1195. 1997
Schoonmaker, J. P., et al., "Effects of age at weaning and implant strategy on growth of steer calves." <i>J. Anim. Sci. (Suppl2)</i> 76:71 (Abstr.). 1998
Seidel, G. E. and L. A. Johnson. "Sexing mammalian sperm -overview." <i>Therio.</i> 52: 1267. 1999
Seidel, G. E., "Insemination of heifers with sexed sperm." <i>Therio.</i> 52:1407. 1999
Seidel, G.E. Jr., "Uterine Horn Insemination of Heifers With Very Low Numbers of Nonfrozen and Sexed Spermatozoa", <i>Atlantic Breeders Cooperative, Theriogenology</i> 48: pp. 1255-1264, (1997)
Seidel, G.E. Jr., Cran, D.G., Herickoff, L.A., Schenk, J.L., Doyle, S.P. and Green, R.D. 1999. Insemination of heifers with sexed frozen or sexed liquid semen. <i>Theriogenology</i> . 51. (in press). abstr.(1999)
Seidel, G.E., Jr., et al, "Artificial Insemination With X-and Y-Bearing Bovine Sperm", <i>Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO; Germplasm and Gamete Physiology Lab, ARS, USDA, Beltsville, MD; Atlantic Breeders Coop, Lancaster, PA; DUO Dairy, Loveland, CO, USA</i> January 1996.
Seidel, G.E., Jr., et al, "Insemination Of Heifers With Very Low Numbers Of Frozen Spermatozoa.", <i>Colorado State University, Fort Collins, Atlantic Breeders Cooperative, Lancaster, PA, DUO Dairy, Loveland, CO, July</i> 1996.
Seidel, Jr., G. E., et al, "Insemination of Holstein Heifers With Very Low Numbers Of Unfrozen Spermatozoa", <i>Colorado State University, Atlantic Breeders Cooperative, (1995)</i>

Seidel, Jr., G.E. et al, "Insemination Of Heifers With Very Low Numbers Of Frozen Spermatozoa", Colorado State University (1996)
Sell, R. S., D. L. Watt, R. D. Little, and T. A. Petry. "Single-calfheifer profitability compared to other north dakota beef production systems." Department of Ag. Eco., North Dakota State University, Ag. Econ. Rpt. 20.
Senger, P.L., Becker, W.C., Davidge, S.T., Hillers, J.K. and Reeves, J.J. 1988. Influence of cornual insemination on conception rates in dairy cattle. J Anim. Sci. 66:3010-3016.
Shackelford, S. D., M. Koohmaraie, and T. L. Wheeler. "Effects of slaughter age on meat tenderness and usda carcass maturity scores of beef females." I. Anim. Sci. 73:3304. 1995
Shelton, J.N. and Moore, N.W. 1967. The response of the ewe to pregnant mare gonadotropin and to horse anterior pituitary extract. J. Reprod. Fert. 14:175 - 177.
Shilova, A.V., Platov, E.M. and Lebedev, S.G. 1976. The use of human chorionic gonadotrophin for ovulation date regulation in mares. VIIIth Int. Congr. On Anim. Repro. and A.I. 204-208.
Shorthose, W. R. and P. V. Harris. "Effect of animal age on the tenderness of selected beef muscles." I. Food Sci. 55:1-. 1990
Silbennann, M., "Honnones and Cartilage. Cartilage: development, differentiation, and growth." pp. 327-368. Academic Press, Inc. 1983
Simon, M., "The effect of management option on the performance of pregnant feedlot heifers." M.S. Thesis. Kansas State University. 1983
Smith, G. C., B. W. Berry, J. W. Savell, and H. R. Cross. "USDA maturity indexes and palatability of beef rib steaks." J. of Food Quality 11 :1. 1988
Smith, G. C., et al., "Relationship of usda maturity groups to palatability of cooked beef." J. of Food Sci. 47:1100. 1982
Squires, E., "Simultaneous Analysis of Multiple Sperm Attributes by Flow Cytometry", Diagnostic Techniques and Assisted Reproductive Technology, The Veterinary Clinics of North America, Equine Practice, Vol. 12, No. 1, April 1996, pp127 - 130.
Squires, E.L, Moran, D.M., Farlin, ME., Jasko, D.J., Keefe, T.J., Meyers, S.A., Figueiredo, E., McCue, P.M. and Jochle, W. 1994. Effect of dose of GnRH analogue on ovulation in mares. Theriogenology. 41:757-769.
Squires, E.L., "Early Embryonic Loss in Equine Diagnostic Ultrasonography", 1 <sup>st</sup> Ed. pp 157-163 Eds Rantanen & McKinnon. Williams and Wilkins, Baltimore, Maryland (1998)
Squires, E.L., et al, "Cooled and frozen stallion semen", Bulletin No. 9, Colorado State University, Ft. Collins, CO. (1999)
Stellflug, J. N., D. K. Ran, R. D. Randel, and E. L. Moody. "Plasma estrogens in peri-parturient cow." Therio 10:269. 1978
Stevenson, J. S., M. W. Smith, J. R. Jaeger, L. R. Corah, and D. G. Lefever. "Detection of estrus by visual observation and radiotelemetry in peripubertal, estrus-synchronized beefheifers." J. Anim. Sci. 74:729. 1996
Story, C. E., R. J. Rasby, R. T. Clark, and C. T. Milton. "Age of calf at weaning of spring-calving beef cows and the effect on cow and calf performance and production economics." J. Anim. Sci. 78:1403. 2000
Sullivan, J.J., Parker, W.G. and Larson, LL. 1973. Duration of estrus and ovulation time in nonlactating mares given human chorionic gonadotropin during three successive estrous periods. J.A.V.M.A. 162:895-898.
Swanson, E. W. "Future research on problems of increasing meat production by early calving." Comm. Eur. Commun., Eur. 5545.1975. The Early Calving of Heifers and its Impact on Beef Production.
Taljaard, T.L., Terblanche, S.J., Bertschinger, H.J. and Van Vuuren, L.J. 1991. The effect of the laparoscopic insemination technique on the oestrus cycle of the ewe. J. S Afr. Vet. Assoc. 62(2):60-61.
Tatum, J. D., G. C. Smith, B. W. Berry, C. E. Murphey, F. L. Williams, and Z. L. Carpenter. "Carcass characteristics, time on feed and cooked beef palatability attributes." J. Anim. Sci. 50:833. 1980
Taylor, C.S., Moore, A.J. Thiessen, R.B. and Bailey, C.M., AFRC Animal Breeding Research Organisation, West Mains Road, Edinburg EH9 3JQ, "Efficiency of Food Utilization in Traditional and Sex-Controlled Systems of Beef Production", pp 401-440.
Taylor, S. C. S., A. J. Moore, R. B. Thiessen, and C. M. Bailey. "Efficiency of food utilization in traditional and sex-controlled systems of beef-production." Animal Production 40:401. 1985
Tervit, H.R., et al., "Successful Culture In Vitro of Sheep and Cattle Ova", Agricultural Research Council, Unit of Reproduction Physiology and Biochemistry, University of Cambridge, 1972, p. 493-497.
Unruh, J. A. "Effects of endogenous and exogenous growth-promoting compounds on carcass composition, meat quality and meat nutritional-value." J. Anim. Sci. 62:1441. 1986

US Application , 09/454,488, entitled "Improved Flow Cytometer Nozzle and Flow Cytometer Sample Handling Methods", filed December 3, 1999.
US Application , 60/238,294, entitled "Hysteroscopic Insemination of Mares" filed October 5, 2000.
US Application , 09/448,643, entitled "Multiple Sexed Embryo Production System for Mammals", filed November 24, 1999.
US Application , 09/511,959 entitled "Methods For Improving Sheath Fluids and Collection Systems For Sex-Specific Cytometer Sorting of Sperm", filed February 23, 2001.
US Application 09/001,394, entitled "Sheath Fluids and Collection Systems for Sex-Specific Cytometer Sorting of Sperm", filed on December 31, 1997, 87 total pages which includes four drawings.
US Application 09/015, 454, entitled "System for Improving Yield of Sexed Embryos in Mammals", filed on January 29, 1998, 59 total pages which includes drawings.
US Application 60/211093, entitled "Integrated System for Herd Management Using Sexed Semen", filed June 12, 2000.
US Application entitled "System For Separating Frozen-Thawed Sperm Cells Into X-Chromosome And Y-Chromosome Bearing Populations", filed November 28, 2000.
US Application Serial Number 60/094,720, entitled "System for Low Dose Insemination of Equines", filed July 30, 1998.
US Application Serial Number 60/113,143, entitled "Equine Insemination System", December 18, 1998.
US Application Serial Number 60/203,089, entitled "Detector System for Resolving Small Differences in Photo-generated Signal", filed May 9, 2000.
US Application Serial Number 60/211093, entitled "Integrated System for Herd Management Using Sexed Semen", filed June 12, 2000.
US Application Serial Number 60/224,050., entitled "Integrated System for Herd Management With Terminal-Cross Program Using Sexed Semen", filed August 9, 2000.
USDA "Official United States standards for grades of carcass beef." Agric, Marketing Serv., USDA .Washington, DC. 1997
Vazquez, J. et al., "Nonsurgical Uterotubal Insemination in the Mare", Proceedings of the 44th Annual Convention of the American Association of Equine Practitioners, Baltimore, Maryland, December 6-9, 1998, Vol. 44, pp 68-69
Vazquez, J., et al., "A.I. in Swine; New Strategy for Deep Insemination with Low Number of Spermatozoa Using a Non-surgical Methodology", 14 <sup>th</sup> International Congress on Animal Reproduction, Vol. 2, Stockholm, July, 2000, p. 289.
Vazquez, J., et al., "Development of a Non-surgical Deep Intra Uterine Insemination Technique", IV International Conference on Boar Semen Preservation, Maryland, August, 1999, p 35 and photo of display board.
Vazquez, J., et al., "Successful Low-Dose Insemination by a Fiberoptic Endoscope Technique in the Sow ", Proceedings Annual Conference of the International Embryo Transfer Society, Netherlands, Theriogenology, Vol. 53, January, 2000, pp. 201.
Vazquez, J., et al., "Hypoosmotic Swelling Test as Predictor of the Membrane Integrity in Boar Spermatozoa", Boar Semen Preservation IV, IVth International Conference on Boar Semen Preservation, Maryland, pp. 263..
Vidament, M., Dupere, A.M., Julienne, P., Evain, A., Noue, P. and Palmer, E. 1997. Equine frozen semen freezeability and fertility field results. Theriogenology. 48:907.
Vincent, B. C., S. D. M. Jones, L. E. Jeremiah, M. A. Price, and J. A. Newman. "Carcass characteristics and meat quality of once-calved heifers." Canadian J. Anim. Sci. 71:311. 1991
Voss, J.L. and Pickett, B.W. 1976. Reproductive management of the broodmare. C.S.U. Exp. Sta. Anim. Reprod. Lab. Gen. Series. Bull. 1-12
Voss, J.L., Pickett, B.W., Burwash, L.D. and Daniels, W.H. 1974. Effect of human chorionic gonadotropin on duration of estrous cycle and fertility of normally cycling, nonlactating mares. J.A.V.M.A. 165:704-706.
Voss, J.L., Squires, B.L., Pickett, B.W., Shideler, R.K. and Eikenberry, D.J. 1982. Effect of number and frequency of inseminations on fertility in mares. J. Reprod. Fertil. Suppl. 32:53-57.
Waggoner, A. W., M. E. Dikeman, I. R. Brethour, and K. E. Kemp. "Performance, carcass, cartilage calcium, sensory and collagen traits of longissimus muscles of open versus 30-month-old heifers that produced one calf." I. Anim. Sci. 68:2380. 1990
Welch G.R., et al., 1994. Fluidic and optical modifications to a FACS IV for flow sorting of X- and Y-chromosome bearing sperm based on DNA. Cytometry 17 (suppl. 7): 74.



Welch, G., et al., "Flow Cytometric Sperm Sorting and PCR to Confirm Separation of X- and Y- Chromosome Bearing Bovine Sperm", <i>Animal Biotechnology</i> , 6 (2), 131-139, 1995, pp 131 - 139.
Wheeler, T. L., L. v. Cundiff, and R. M. Koch. "Effect of marbling degree on beef palatability in Bos- Taurus and Bos-Indicus cattle." <i>J. Anim. Sci.</i> 72:3145. 1994
Wickersham, E. W. and L. H. Schultz. "Influence of age at first breeding on growth, reproduction, and production of well-fed holstein heifers." <i>J. Dairy Sci.</i> 46:544. 1963
Wilson, C.G., Downie, C.R., Hughes, J.P. and Roser, J.F. 1990. Effects of repeated hCG injections on reproductive efficiency in mares. <i>Eq. Vet. Sci.</i> 4:301-308.
Wilson, M.S. 1993. Non-surgical intrauterine artificial insemination in bitches using frozen semen. <i>J.Reprod. Fert Suppl.</i> 47:307-311.
Woods, J. and Ginther, O.J. 1983. Recent studies related to the collection of multiple embryos in mares. <i>Theriogenology.</i> 19:101 - 108.
Woods, J., Bergfelt, D.R. and Ginther, O.J. 1990. Effects of time of insemination relative to ovulation on pregnancy rate and embryonic-loss rate in mares. <i>Eq. Vet. J.</i> 22(6):410-415.
XP-002103478, File Biosis, one page.

In addition, as to each term used it should be understood that unless its utilization in this application is inconsistent with such interpretation, common dictionary definitions should be understood as incorporated for each term and all definitions, alternative terms, and synonyms such as contained in the Random House Webster's Unabridged Dictionary, second edition are hereby incorporated by reference. However, as to each of the above, to the extent that such information or statements incorporated by reference might be considered inconsistent with the patenting of this/these invention(s) such statements are expressly not to be considered as made by the applicant(s).

In addition, unless the context requires otherwise, it should be understood that the term "comprise" or variations such as "comprises" or "comprising", are intended to imply the inclusion of a stated element or step or group of elements or steps but not the exclusion of any other element or step or group of elements or steps. Such terms should be interpreted in their most expansive form so as to afford the applicant the broadest coverage legally permissible in countries such as Australia and the like.

Thus, the applicant(s) should be understood to have support to claim at least: i) each of the staining, separation, isolation, insemination, or fertilization procedures as herein disclosed and described, ii) the related methods disclosed and described, iii) similar, equivalent, and even implicit variations of each of these devices and methods, iv) those



alternative designs which accomplish each of the functions shown as are disclosed and described, v) those alternative designs and methods which accomplish each of the functions shown as are implicit to accomplish that which is disclosed and described, vi) each feature, component, and step shown as separate and independent inventions, vii) the applications  
5 enhanced by the various systems or components disclosed, viii) the resulting products produced by such systems or components, ix) methods and apparatuses substantially as described hereinbefore and with reference to any of the accompanying examples, and x) the various combinations and permutations of each of the elements disclosed.

10       The claims set forth in this specification are hereby incorporated by reference as part of this description of the invention, and the applicant expressly reserves the right to use all of or a portion of such incorporated content of such claims as additional description to support any of or all of the claims or any element or component thereof, and the applicant further expressly reserves the right to move any portion of or all of the incorporated content of such  
15 claims or any element or component thereof from the description into the claims or vice-versa as necessary to define the subject matter for which protection is sought by this application or by any subsequent continuation, division, or continuation-in-part application thereof, or to obtain any benefit of, reduction in fees pursuant to, or to comply with the patent laws, rules, or regulations of any country or treaty, and such content incorporated by  
20 reference shall survive during the entire pendency of this application including any subsequent continuation, division, or continuation-in-part application thereof or any reissue or extension thereon.

## VI. CLAIMS

I claim:

- 5 1. A method of staining sperm cells collected from mammals, comprising the steps of:
  - a. collecting semen from a male mammal;
  - b. incubating sperm cells contained within said semen in a concentration of Hoechst 33342 stain of greater than 40 micro-molar;
  - c. establishing the temperature at which said sperm cells in said concentration of  
10 Hoechst 33342 stain are incubated between about 30 degrees centigrade and about 40 degrees centigrade;
  - d. adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes; and
  - 15 e. staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%.
- 20 2. A method of staining sperm cells collected from mammals as described in claim 1, wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.
3. A method of staining sperm cells collected from mammals as described in claim 2,  
25 wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.
4. A method of staining sperm cells collected from mammals as described in claim 2,  
30 wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is 224 micro-molar.

5. A method of staining sperm cells collected from mammals as described in claim 2, wherein said male mammal comprises said bovid and said concentration of Hoechst 3342 stain is 2240 micro-molar.

5

6. A method of staining sperm cells collected from mammals as described in claim 4, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.

10

7. A method of staining sperm cells collected from mammals as described in claim 5, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

15

8. A method of staining sperm cells collected from mammals as described in claim 1, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

20

25

9. A method of staining sperm cells collected from mammals as described in claim 8, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.

30

10. A method of staining sperm cells collected from mammals as described in claims 1,

2, 6, 7, 8, or 9, further comprising the step of freezing said semen.

11. A method of staining sperm cells collected from mammals as described in claim 10, further comprising the step of thawing said semen.

5

12. A method of staining sperm cells collected from mammals as described in claim 11, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.

10

13. A method of staining DNA within frozen-thawed sperm cells, comprising the steps of:

15

- a. collecting semen containing sperm cells from a male mammal;
- b. freezing said semen containing said sperm cells;
- c. thawing said semen containing said sperm cells;
- d. combining frozen-thawed semen with Hoechst 33342 stain;
- e. establishing a concentration of said Hoechst 33342 stain between about 200 micro-molar and about 2500 micro-molar;
- f. adjusting the temperature of said frozen-thawed semen in said concentration of Hoechst 33342 stain to between about 30 degrees Centigrade and about 40 degrees Centigrade; and
- g. adjusting the duration of time said frozen-thawed semen in said concentration of Hoechst 33342 stain incubates to between about 50 minutes and 200 minutes, whereby DNA within sperm cells contained in said frozen-thawed are stained with sufficient uniformity to differentiate between X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells on the basis of magnitude of fluorescence.

20

25

30

14. A method of staining sperm cells collected from mammals as described in claim 13,

wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

15. A method of staining sperm cells collected from mammals as described in claim 14,  
5 wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.
16. A method of staining sperm cells collected from mammals as described in claim 14,  
10 wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is 224 micro-molar.
17. A method of staining sperm cells collected from mammals as described in claim 14,  
15 wherein said male mammal comprises said bovid and said concentration of Hoechst 3342 stain is 2240 micro-molar.
18. A method of staining sperm cells collected from mammals as described in claim 14,  
20 wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.
19. A method of staining sperm cells collected from mammals as described in claim 17,  
25 wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.
20. A method of staining sperm cells collected from mammals as described in claims 13,  
30 15, 16, 17, 18, 19, or 20, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of

fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

5

21. A method of staining sperm cells collected from mammals as described in claim 20, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.

10 22. A method of staining sperm cells collected from mammals as described in claim 20, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow  
15 cytometer.

23. A method of generating mammalian embryos, comprising the steps of:

- a. collecting semen from a male mammal;
- b. combining said semen from said male mammal with an amount of Hoechst  
20 33342 stain;
- c. establishing a concentration of Hoechst 33342 stain combined with said semen to a concentration between about 40 micro-molar and about 2500 micro-molar;
- d. adjusting the temperature at which said sperm cells are incubated with said  
25 Hoechst 33342 stain between about 30 degrees centigrade and about 40 degrees centigrade;
- e. adjusting the duration of time said sperm cells are incubated in said concentration of Hoechst 33342 between about 60 minutes and about 200 minutes;
- f. staining DNA within sperm cells contained in said semen with said Hoechst  
30 33342 stain; and

g. fertilizing oocytes with stained sperm cells,  
whereby increasing the concentration of Hoechst 33342 stain and decreasing  
the duration of time said sperm cells are incubated in said concentration of  
Hoechst 33342 stain increases the percentage of said mammalian embryos  
5 produced.

24. A method of staining sperm cells collected from mammals as described in claim 23,  
wherein said male mammal is selected from the group of mammals consisting of  
primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

10

25. A method of staining sperm cells collected from mammals as described in claim 24,  
wherein said male mammal comprises said bovid and said concentration of Hoechst  
33342 stain is between about 200 micro-molar and about 2500 micro-molar.

15 26. A method of staining sperm cells collected from mammals as described in claim 24,  
wherein said male mammal comprises said bovid and said concentration of Hoechst  
33342 stain is 224 micro-molar.

20 27. A method of staining sperm cells collected from mammals as described in claim 24,  
wherein said male mammal comprises said bovid and said concentration of Hoechst  
3342 stain is 2240 micro-molar.

25 28. A method of staining sperm cells collected from mammals as described in claim 26,  
wherein said step of adjusting a duration of time said sperm cells are incubated with  
said concentration of Hoechst 33342 stain between about 50 minutes and about 200  
minutes comprises adjusting said duration of time said sperm cells are incubated with  
said concentration of Hoechst 33342 stain to about 190 minutes.

30 29. A method of staining sperm cells collected from mammals as described in claim 27,  
wherein said step of adjusting a duration of time said sperm cells are incubated with  
said concentration of Hoechst 33342 stain between about 50 minutes and about 200

minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

30. A method of staining sperm cells collected from mammals as described in claims 23, 25, 26, 27, 28, 29, or 30, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.
31. A method of staining sperm cells collected from mammals as described in claim 30, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.
32. A method of staining sperm cells collected from mammals as described in claim 30, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.
33. A method of staining sperm cells collected from mammals as described in claim 32, further comprising the step of isolating differentiated X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells into separate collection elements.
34. A method of staining sperm cells collected from mammals as described in claim 33, wherein said step of isolating differentiated X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells into separate collection elements comprises isolating Y-chromosome bearing sperm cells into a separate collection element at a



rate of about 1000 per second.

35. A method of staining sperm cells collected from mammals as described in claim 33, wherein said step of isolating differentiated X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells into separate collection elements comprises isolating X-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.
36. A method of generating mammalian embryos as described in claims 23, further comprising the step of freezing said semen.
37. A method of generating mammalian embryos as described in claims 30, further comprising the step of freezing said semen.
38. A method of generating mammalian embryos as described in claim 36, further comprising the step of thawing said semen.
39. A method of generating mammalian embryos as described in claim 37, further comprising the step of thawing said semen.
40. A flow cytometer system for isolating desired sperm cells, comprising:
- a. sperm cells obtained by thawing previously frozen semen, wherein said sperm cells are incubated with a concentration of Hoechst 33342 stain between about 200 micro-molar and about 2500 micro-molar until DNA within said sperm cells are stained with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%;
  - b. a sperm cell source that supplies said sperm cells to a flow cytometer;
  - c. a sheath fluid source that creates a sheath fluid environment within said flow cytometer in which said sperm cells are entrained;

- d. a nozzle through which said sperm cells pass while entrained in said sheath fluid environment;
  - e. an oscillator that acts upon said sheath fluid as it passes through said nozzle;
  - f. a sperm cell sensing system responsive to said sperm cells;
  - 5 g. a separation discrimination system that acts to separate said sperms cells having a desired characteristic; and
  - h. a containment element into which said sperm cells having said desired characteristic are collected.
- 10 41. A flow cytometer system for isolating desired sperm cells as described in claim 40, wherein said sperm cells obtained by thawing previously frozen semen are obtained from male mammals selected from the group consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.
- 15 42. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.
- 20 43. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is about 224 micro-molar.
- 25 44. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 3342 stain is 2240 micro-molar.
- 30 45. A flow cytometer system for isolating desired sperm cells as described in claim 42, further comprising a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes.

46. A flow cytometer system for isolating desired sperm cells as described in claim 43, wherein said duration of time about 190 minutes.
47. A flow cytometer system for isolating desired sperm cells as described in claim 44,  
5 wherein said duration of time about 60 minutes.
48. A flow cytometer system for isolating desired sperm cells as described in claims 40,  
42, 43, 45, or 48, wherein said X-chromosome bearing sperm cells to be  
differentiated from Y-chromosome bearing sperm cells based upon the magnitude of  
10 fluorescence at a rate of greater than about 85% is a rate selected from the group  
consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,  
98%, or 99%.
49. A flow cytometer system for isolating desired sperm cells as described in claim 48,  
15 further comprising a collection element into which differentiated X-chromosome  
bearing sperm cells are isolated.
50. A flow cytometer system for isolating desired sperm cells as described in claim 48,  
further comprising a collection element into which differentiated Y-chromosome  
20 bearing sperm are isolated.
51. A flow cytometer system for isolating desired sperm cells as described in claim 49,  
further comprising a rate at which X-chromosome bearing sperm cells are isolated  
greater than about 1000 per second.
- 25 52. A flow cytometer system for isolating desired sperm cells as described in claim 50,  
further comprising a rate at which Y-chromosome bearing sperm cells are isolated  
greater than about 1000 per second.
- 30 53. A method of generating mammalian embryos as described in claim 40, further  
comprising the step of freezing said semen.

54. A method of generating mammalian embryos as described in claims 51, further comprising the step of freezing said semen.
- 5 55. A method of generating mammalian embryos as described in claim 53, further comprising the step of thawing said semen.
56. A method of generating mammalian embryos as described in claim 54, further comprising the step of thawing said semen.
- 10 57. A method of producing a mammal having a predetermined sex comprising the steps of:
- a. collecting semen from a male mammal;
  - b. freezing said semen;
  - 15 c. thawing said semen;
  - b. determining the sex characteristic of a plurality of sperm cells contained within said frozen-thawed semen;
  - c. separating said sperm cells according to the determination of their sex characteristic;
  - 20 d. isolating sperm cells separated according to the determination of their sex in a collection element;
  - d. establishing an artificial insemination sample from said sperm cells isolated in said collection element;
  - e. inserting said artificial insemination sample into a female mammal of the same species from which said semen was collected;
  - 25 f. fertilizing at least one egg within said female mammal; and
  - g. producing an offspring mammal of the desired sex.
58. A method of producing a mammal having a predetermined sex as described in claim 30 57, wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

59. A method of producing a mammal having a predetermined sex as described in claim 58, further comprising the step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar.

5

60. A method of producing a mammal having a predetermined sex as described in claim 59, wherein said step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar comprises staining of sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%.

10

61. A method of producing a mammal having a predetermined sex as described in claim 59, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.

15

62. A method of producing a mammal having a predetermined sex as described in claim 61, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is 224 micro-molar.

20

63. A method of producing a mammal having a predetermined sex as described in claim 61, wherein said male mammal comprises said bovid and wherein said concentration of Hoechst 3342 stain is 2240 micro-molar.

25

64. A method of producing a mammal having a predetermined sex as described in claim 61, further comprising the step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes.

30

65. A method of producing a mammal having a predetermined sex as described in claim

62, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.

5

66. A method of producing a mammal having a predetermined sex as described in claim 63, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

10

67. A method of producing a mammal having a predetermined sex as described in claims 57, 58, 61, 62, 63, 64, 65, or 66, wherein said step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar comprises staining of sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

15

20

68. A method of producing a mammal having a predetermined sex as described in claim 67, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.

25

69. A method of staining sperm cells collected from mammals as described in claim 68, wherein said step of isolating sperm cells separated according to the determination of their sex in a collection element comprises isolating Y-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.

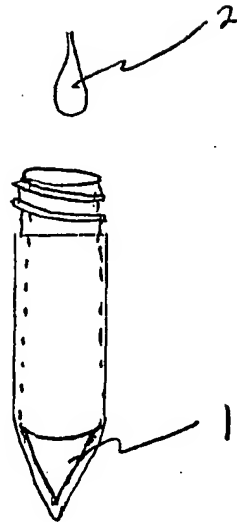
30

70. A method of staining sperm cells collected from mammals as described in claim 68, wherein said step of isolating sperm cells separated according to the determination of their sex in a collection element comprises isolating X-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.
71. A method of producing a mammal having a predetermined sex as described in claim 57, further comprising the step of limiting the number of isolated sperm cells in said artificial insemination sample to about 10% to about 50% of the number of said sperm cells relative to a typical unseparated artificial insemination sample.
72. A method of producing a mammal having a predetermined sex as described in claim 58, wherein said mammal comprises said bovid and wherein said artificial insemination sample has the number of isolated sperm cells limited to about one million to three million.
73. A method of producing a mammal having a predetermined sex as described in claim 58, wherein said mammal is a bovid and wherein said artificial insemination sample has the number of isolated sperm cells limited to between about one-hundred and fifty thousand and about one million.
74. A method of producing a mammal having a predetermined sex as described in claim 57, wherein said mammal comprises said equid and wherein said artificial insemination sample has the number of isolated sperm cells limited to between about forty million and about one hundred million.
75. A method of producing a mammal having a predetermined sex as described in claims 71, 72, 73 or 74, further comprising the step of creating superovulation in said female mammal to create at least two eggs comprising the step of using an ovulatory pharmaceutical to cause multiple eggs to be produced, and wherein said ovulatory pharmaceutical is injected in half day increments between any of days 2 and 18 of

the estrus cycle.



FIG 1



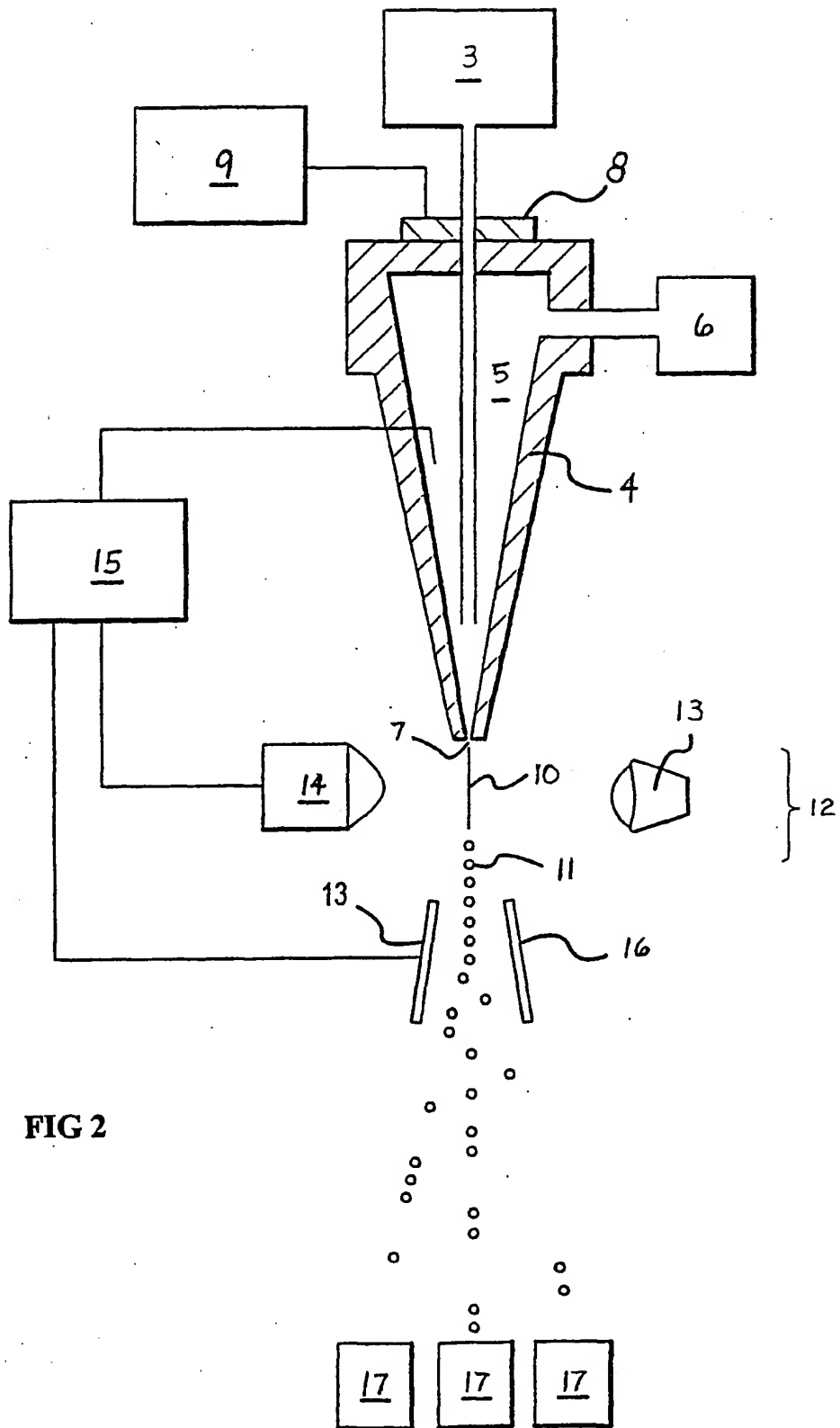


FIG 2

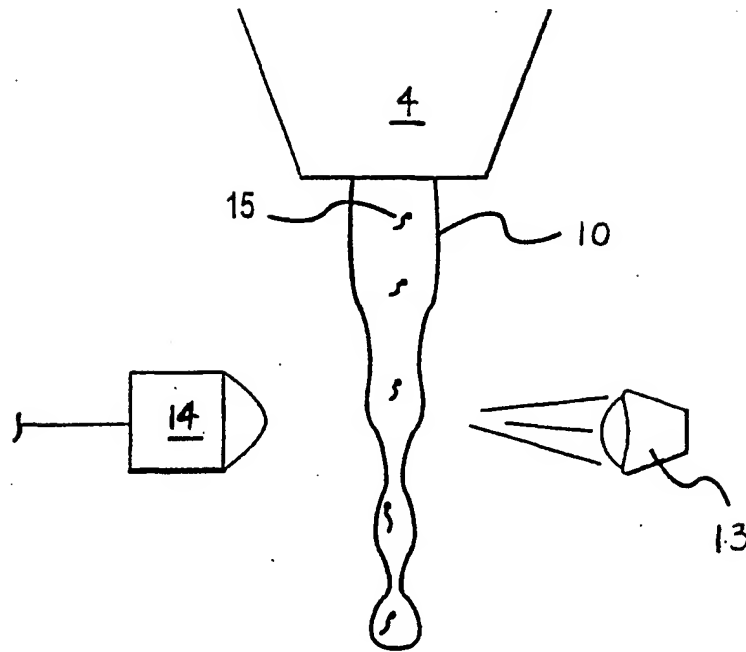


FIG 3

